SWIMMING PERFORMANCE AND ENERGY HOMEOSTATIC EFFECTS OF URANIUM MILL EFFLUENT EXPOSURE IN SMALL-BODIED FISH

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By

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ABSTRACT

Previous studies at the Key Lake uranium mill (Saskatchewan, Canada) suggested the complex effluent discharged alters energetic stores of resident fish species. A second study at the same site demonstrated certain fish from lakes downstream of the mill produce larvae with elevated incidence of developmental deformities. The mechanisms by which energy homeostasis is affected in fish downstream of the Key Lake uranium mill are unknown, and the effects of deformities and altered metabolism on swimming ability have not been explored. Therefore, the overall objective of this thesis was to investigate whether effluent exposed fish exhibited differences in swimming performance and energy homeostasis.

To achieve this objective two experiments were conducted. In the first experiment juvenile spottail shiner (*Notropis hudsonius*) were collected from a lake downstream of the Key Lake uranium mill, and compared to fish collected from a nearby reference lake. In the second experiment larvae were collected from laboratory raised fathead minnow (*Pimephales promelas*) exposed to 5% diluted uranium mill effluent or control (dechlorinated municipal) water, and reared in the same treatments to 60 days post hatch (dph). No gross deformities were observed in any fish, and only shiner collected from the exposure lake in the field experiment had enlarged heart ventricles relative to body size compared to fish from the reference lake. Swimming performance was similar between shiner from the exposure and reference lakes in the field study, but effluent exposure impaired swimming ability in 60 dph fathead minnow in the laboratory experiment compared to fish from the control water treatment. After swimming performance tests fish were considered fatigued and metabolic endpoints were compared to non-fatigued fish. In both non-fatigued and fatigued shiner, liver glycogen was significantly greater in fish collected from the exposure lake compared to the reference lake. There was no difference in liver

triglycerides in non-fatigued shiner between lakes, but liver triglycerides decreased after swimming in the field study reference fish. Muscle energy stores were unaffected by site or swimming in the field experiment. Conversely, whole body triglycerides and glycogen were similar between treatments in non-fatigued fathead minnow in the laboratory experiment. Swimming significantly decreased whole body triglycerides in fathead minnow from both treatments, but whole body glycogen was unaffected. In the field experiment blood endpoints (hematocrit, plasma glucose, lactate) in fatigued and non-fatigued shiner from both lakes further supported the possibility of altered intermediary metabolism or blunted stress response in fish downstream of the Key Lake uranium mill. In the field study, shiner muscle citrate synthase activity (an indicator of tissue aerobic capacity) was similar between lakes, but muscle β–hydroxyacyl coenzyme A dehydrogenase activity (an indicator of tissue lipolytic capacity) was elevated. In contrast, laboratory fathead minnow whole body β–hydroxyacyl coenzyme A dehydrogenase activity was similar between treatments, but citrate synthase activity was significantly lower in fathead minnow from the 5% effluent treatment.

In summary, shiner from the exposure lake in the field experiment had similar swimming endurance and greater energy stores compared to fish from the reference lake, despite metabolic alterations. Fathead minnow from the 5% effluent treatment in the laboratory experiment had reduced swimming endurance that was matched by reduced whole body citrate synthase activity, but no other metabolic alterations were observed. Therefore, effluent exposure caused metabolic alterations in both fathead minnow and shiner, but specific effects between experiments were inconsistent. Overall, the physiological significance of the metabolic and swimming effects of effluent exposure is unclear, but suggests discharged effluent has the potential to negatively affect wild fish survivability.

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LIST OF ABBREVIATIONS

A	Atrium
ATP	Adenosine triphosphate
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
BA	Bulbus arteriosus
BL	Body length
CCME	Canadian Council of Ministers of the Environment
CoA	Coenzyme A
CF	Condition factor
CS	Citrate synthase
DA	Dorsal aorta
Dph	Days post hatch
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EC_{50}	50% Effective concentration
EDTA	Ethylenediaminetetraacetic acid
$FADH_2$	Flavin adenine dinucleotide
FHM	Fathead minnow
GTP	Guanosine triphosphate
HOAD	β-hydroxyacyl coenzyme A dehydrogenase
HSI	Hepatosomatic index
ICP-MS	Inductively coupled plasma-mass spectrometry
LC_{50}	50% Lethal concentration

M_{O2}	Mean oxygen consumption
NADH	Nicotinamide-adenine dinucleotide
SEM	Standard error of the mean
Se-met	Selenomethionine
t_i	Increment time length in Critical swimming speed test
t_f	The duration of the last velocity increment until fatigue in Critical swimming speed test
TNB	5-thio-2-nitrobenzoic acid
U_{crit}	Critical swimming speed
U_{max}	Maximum (burst) swimming speed
U_{opt}	Optimal swimming speed
V	Ventricle
V_i	Velocity increase per time increment in Critical swimming speed test
V_p	Final velocity swam in Critical swimming speed test
WSS	Winter stress syndrome

PREFACE

Chapter 1 is a general introduction while Chapter 2 contains unpublished data from method development of two enzyme assays used in this thesis. Chapters 3 and 4 of this thesis are organized as manuscripts for publication in scientific journals. Therefore, there is some repetition of introductions, materials and methods throughout each data chapter. Chapter 3 was submitted to Ecotoxicology and Environmental Safety and Chapter 4 was submitted to Comparative Physiology and Biochemistry C.

CHAPTER 1
1.0 GENERAL INTRODUCTION

1.1 The Key Lake uranium milling operation and environmental effects

1.1.1 Location and the milling process

The Key Lake uranium milling operation (57°13'N, 105°38'W) is located in the Athabasca region of north-central Saskatchewan, approximately 600 km north of Saskatoon (Figure 1.1). This region is classified as a northern coniferous forest biome, also referred to as the boreal forest or the boreal shield ecozone (Larsen, 1980). This area encompasses distinctive terrain, characterized by northern mixed wood forests, mostly comprised of conifers, with oligotrophic water bodies. Water bodies can undergo rapid temperature change as soon as surface ice melts, especially in shallow lakes, while organisms in this region experience long, cold winters, and relatively short growing seasons. With increasing interest in the effects of climate change and environmental impact of human activities there is growing concern about impacts on northern water bodies, which could be especially sensitive to environmental stressors, such as acidification, eutrophication, contaminants and/or climate change (Schindler and Smol, 2006; Keller, 2007).

Cameco Corporation owns and operates the Key Lake uranium milling operation. Ore processed at this mill originates from the McArthur river uranium mine, located approximately 70 km north east of the mill site. Open pit mining occurred at the Key Lake site from 1982 to 2002, while milling of the McArthur river uranium deposit commenced in 2000 and is expected to continue for approximately 25 years (Golder Associates, 2008). Raw ore is shipped daily from the McArthur mine site to the Key Lake milling operation in order to liberate and extract uranium compounds to form yellowcake (U_3O_8), the final mill product. The milling process involves various industrial chemicals, including sulphuric acid, organics (kerosene, isodecanol,

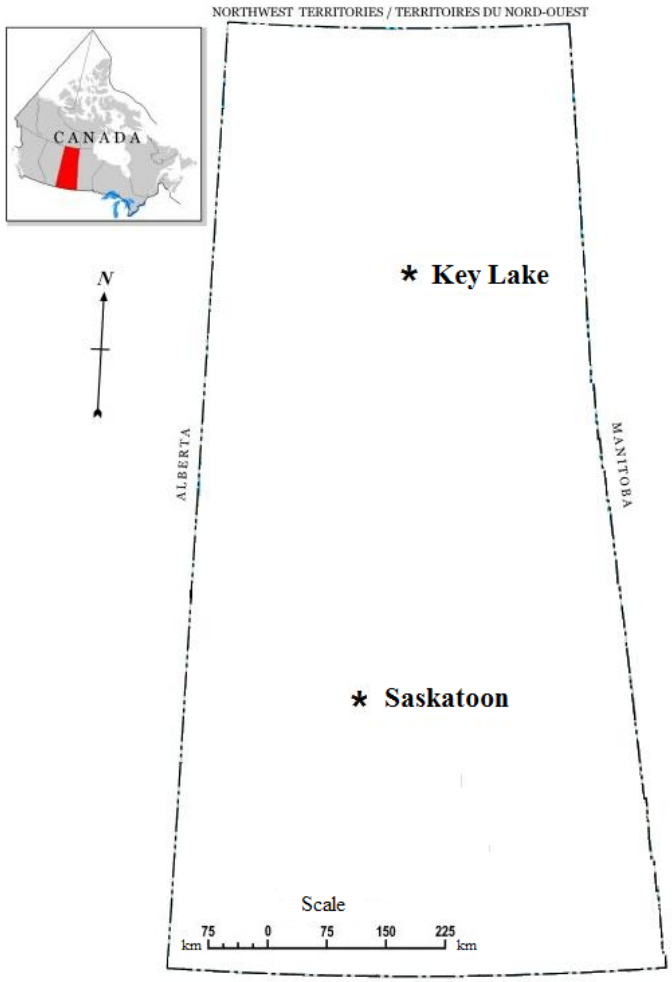

Figure 1.1 Map of approximate Key Lake uranium mill location (57°13’N, 105°38’W) within Saskatchewan, Canada. Figure modified from Natural Resources Canada (2003).

trialkylamines), and ammonia (Robertson and Liber, 2007; Golder Associates, 2008), which can also liberate trace elements associated with the uranium ore. Through the effluent treatment process, the majority of remaining trace metals and metalloids are removed from effluent, which is also pH neutralized, before it is discharged to ensure provincial and federal water quality guidelines are met. However, not all trace elements, processing chemicals (ammonia, organics), and associated ions are wholly removed from effluent during treatment processes. As a result, elevated concentrations of these contaminants accumulate in downstream water bodies. Two different streams of effluent are generated from yellowcake production: 1) dewatering effluent, yielded directly from milling processes, which is discharged into the McDonald Lake drainage system (Figure 1.2B); and 2) treated mill effluent, composed of remaining ore constituents, which is discharged into the David Creek drainage system (Figure 1.2A; Golder Associates, 2008).

Earlier water chemistry data suggested discharged treated mill effluent, rather than dewatering effluent, had the greatest potential to adversely affect downstream environments (Golder Associates, 2008). Therefore, the David Creek drainage system has been the subject of ongoing environmental investigations. In 2006, the Key Lake uranium mill discharged approximately 1,613,349 m^3 treated effluent into Wolf Lake (Figure 1.2A; Golder Associates, 2008), which drains into Fox Lake, leading to Unknown Lake, and then Delta Lake (Figure 1.2A). As a result, an effluent gradient is observed in these lakes, and in 2003 effluent accounted for approximately 72% of Wolf Lake outflow, which decreased to approximately 28% within Delta Lake (Golder Associates, 2008). Delta Lake has been of particular interest in ongoing environmental studies as it is the first water body downstream of the Key Lake uranium mill that supports fish populations (Golder Associates, 2008) although low numbers of burbot

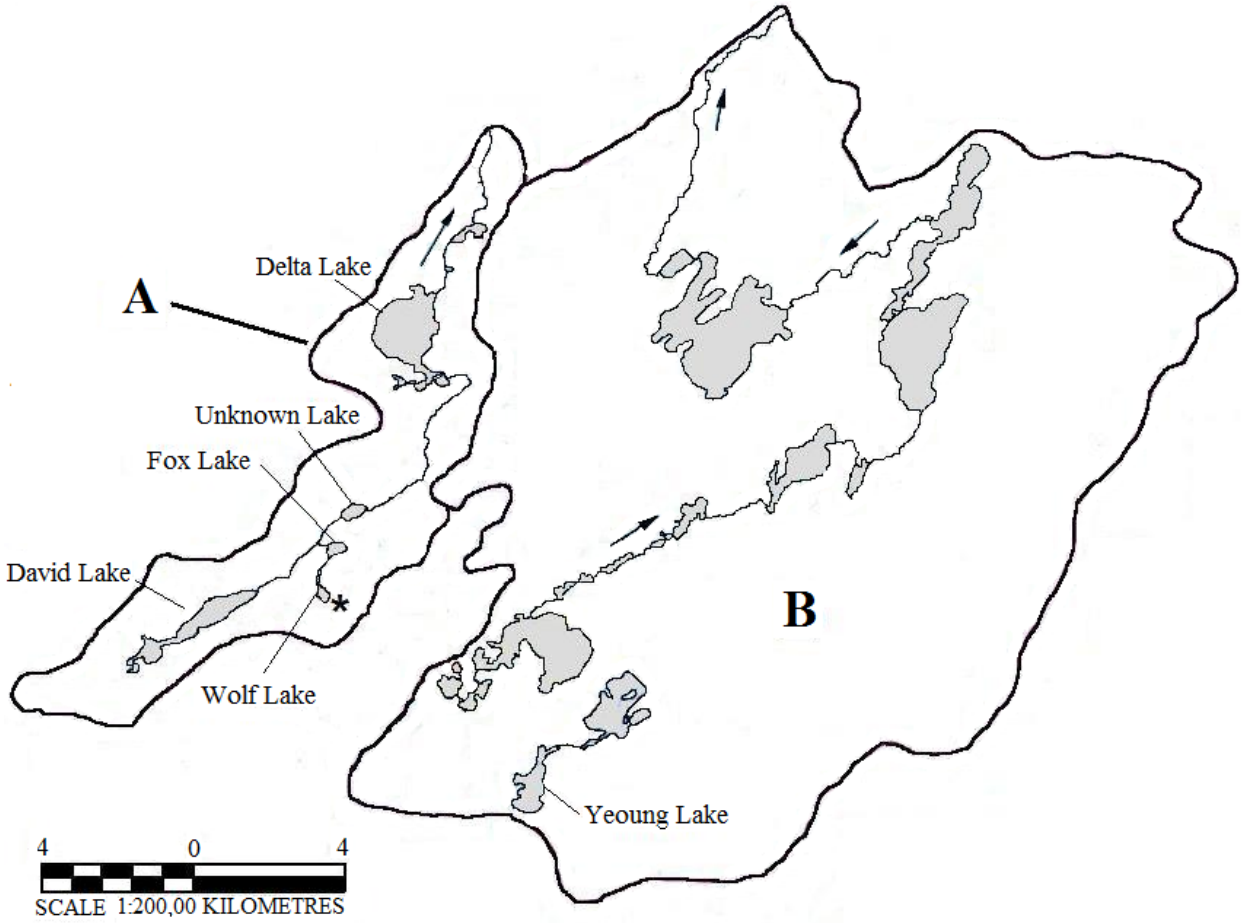

Figure 1.2 Map of reference (Yeoung Lake) and exposure (Delta Lake) sites within watersheds associated with the Key Lake uranium mill, Saskatchewan. Figure modified from Golder Associates (2008). (A) David Creek drainage system; (B) McDonald Lake drainage system. Asterisk (*) indicates effluent discharge point. Direction of water flow is indicated with thin arrows and lakes are shown in grey. Thick lines indicate watershed boundaries.

(*Lota lota*; Bennett and Janz, 2007) and juvenile northern pike (*Esox lucius*; Muscatello et al., 2006) have been captured in Unknown Lake. No studies have quantified fish populations in Delta Lake and limited information exists on the population effects of effluent exposure in fish species in this water body. Boat electrofishing sampling in 2007 yielded significantly lower catch per unit effort values for small bodied fish species in Delta Lake compared to 2004 sampling (Golder Associates, 2008). Although this could signify negative population effects of discharged effluent, other factors could also contribute to lower values, including seasonal movement of schooling fish (Golder Associates, 2008). Furthermore, in the same 2007 study catch per unit effort for large fish species (*e.g*. pike) was similar between Delta Lake and Yeoung Lake (a reference lake) although low numbers were observed in both lakes. Therefore, the population effects of discharged milling effluent are unclear. However, it has been established that Delta Lake resident fish are chronically exposed to low effluent concentrations and studies have demonstrated various effects of exposure in sampled fish (Muscatello et al., 2006; Bennett and Janz, 2007; Golder Associates, 2008; Kelly and Janz, 2008)

In order to adequately assess changes in lakes downstream of the Key Lake uranium mill, a number of suitable reference lakes have been identified for comparative purposes. Previous studies employed David Lake as the reference site, as it is located upstream of the mill and contained similar organism populations as downstream lakes (Figure 1.2A; Bennett and Janz, 2007; Kelly and Janz, 2008). More recently, Yeoung Lake, a reference lake within the MacDonald Lake drainage, was identified by Golder Associates (2008) as a suitable additional reference lake to the David Creek drainage for sediment, benthic invertebrate communities, and small body fish endpoints in environmental monitoring programs. As a result, Yeoung Lake is

considered ecologically similar to Delta Lake, and has been used in recent studies as the sole reference lake in comparison to Delta (Phibbs et al., 2011; Hauck and Janz, unpublished).

1.1.2 Toxicological investigations of effects of Key Lake mill effluent in wild fish

Discharged effluent directly contributes to elevated environmental concentrations of several trace elements (most notably arsenic, cadmium, iron, molybdenum, manganese, nickel, selenium and uranium), ions and nutrients (Ca^{2+}, Cl^{-}, Na^{+}, K^{+}, ammonium, sulphate) in lakes downstream of the mill compared to reference lakes (Bennett and Janz, 2007; Golder Associates, 2008; Kelly and Janz, 2008). As a result, fish in lakes downstream of the mill can incur varying levels of toxicity depending on site. Laboratory-reared larval fathead minnow (*Pimephales promelas*; <24 hours post hatch) placed in Fox (high effluent exposure) or Unknown (medium effluent exposure) Lakes for seven days experienced significantly higher mortality than fish in David Lake (Pyle et al., 2001). Although many elements were elevated in the exposure lakes, mortality was statistically most strongly associated with dissolved molybdenum concentration (Pyle et al., 2001). As aqueous molybdenum has relatively low toxicity to fish, the authors instead suggest dietary selenium was the source of toxicity (Pyle et al., 2001). Selenium was further correlated with elevated deformities and edema in larval northern pike from adults collected from Delta Lake (Muscatello et al., 2006). Because Wolf and Fox Lakes are unable to support fish populations (due to the high toxicity of concentrated treated mill effluent), the sublethal effects of low/medium effluent exposure have been more broadly investigated in fish from Unknown and Delta Lakes. In particular, recent studies have highlighted interesting sublethal effects of chronic effluent exposure in wild fish from these sites, especially effects on energy stores. One study found pike and burbot collected from both Unknown and Delta Lakes had greater liver triglyceride levels compared to fish from a reference lake (David Lake; Bennett

and Janz, 2007). Similarly, a follow up study demonstrated overwintering juvenile northern pike from Delta Lake possessed higher muscle glycogen and higher liver triglycerides compared to fish from the reference lake (Kelly and Janz, 2008). However, no difference was observed in prey triglyceride levels in this same study. Overall, previous studies in pike from Delta Lake demonstrated clear signs of toxicity (*e.g*. elevated incidence of larval mortalities) due to effluent exposure, but juvenile pike also had greater energy stores than fish from reference lakes, which did not appear to be related to greater food web productivity. It is unclear which effluent constituent(s) is/are responsible for these effects.

1.1.3 Challenges of assessing sublethal effects of effluent exposure in fish

While the acute toxic effects of Key Lake uranium mill effluent exposure have been investigated (Pyle et al., 2001; Golder Associates, 2005), the sublethal effects of more chronic exposures in fish are unclear (Bennett and Janz, 2007; Kelly and Janz, 2008). Complex contaminant mixtures can pose difficulties when assessing environmental effects. Uranium milling effluent discharged into the David Creek drainage system contains various families of environmental contaminants (metals, organics, nutrients, ions, pH-altering substances) and resident organisms can experience numerous routes of exposure. Furthermore, effects are difficult to predict or interpret as complex mixtures can cause effects that surpass or counteract additive effects of individual contaminants (Altenburger et al., 2003; Forbes et al., 2006).

Organisms may experience effects of exposure even when discharged effluent meets federal and provincial water quality criteria. Guidelines derived from ecological risk assessment generally focus on protecting populations, communities and ecosystems (Forbes et al., 2006). Furthermore, regulatory guidelines for environmental contaminant concentrations are traditionally based on survival tests, in addition to reproductive and developmental endpoints

(reviewed by Scott and Sloman, 2004; CCME, 2007). While these endpoints are considered general indicators of fitness (and thus, can be used to reasonably assess population health), the relationship between these variables can be inconsistent and non-linear (Forbes et al., 2006).

Water quality guidelines are important as they protect organisms against contaminant-induced physiological death (mortality), but generally fail to address the organism's "ecological death" at lower contaminant concentrations (Scott and Sloman, 2004). In other words, at lower contaminant concentrations, an organism may not be explicitly harmed, but may experience behavioural or metabolic effects that prevent the organism from functioning normally in its environment. Based on these observations, it is plausible fish downstream of the Key Lake mill exhibit behavioural or intermediary metabolic effects that inadvertently affect individual survivability. Beyond basic metabolic investigations (Bennett and Janz, 2007; Kelly and Janz, 2008), little research has occurred investigating these effects. However, by assessing biochemical, tissue and whole organism-level effects, it could be possible to derive guidelines that better protect populations and ecosystems as a whole.

1.2 Swimming performance in fish

Swimming is integral to fish survival and plays a role in many fish behaviours. An individual's survivability greatly depends on swimming ability, influencing feeding, predator evasion, migration, and mating (Beaumont et al., 1995; Drucker, 1996). Swimming performance in fish has been under investigation for many years, but ecologically relevant quantification remains challenging. As a result, various test protocols have been introduced to characterize swimming. Most currently employed protocols involve the placement of individual fish in a chamber containing static or flowing oxygenated water. The fish then swims within the chamber for specific durations of time (most fish exhibit rheotropism and instinctually swim against a

current when applied), with water current velocity influencing swimming intensity. In general, these protocols evaluate swimming modes that can be broadly divided into three categories: sustained, burst, and prolonged (Beamish, 1978).

1.2.1 Swimming performance classifications

During sustained swimming fish maintain locomotion for prolonged periods of time without fatiguing, possibly over a period of days or months (Hammer, 1995). Sustained activity beyond maintenance metabolism recruits aerobic metabolic pathways that generate ATP. While anaerobic metabolic processes could be employed during periods in which the cardiovascular system fails to sufficiently oxygenate tissue, sustained activity is almost entirely aerobic. Anaerobic intramuscular fuels (free ATP and creatine phosphate) are minimally utilized during sustained swimming and while some anaerobic by-products may be generated (*i.e*. lactate), production generally falls into equilibrium with recovery (Jobling, 1994). It is unclear the degree to which anaerobic metabolic processes contribute, if at all, to this sustained activity level in salmonids. However, indirect evidence in goldfish (*Carassius auratus*) suggests anaerobic pathways may partly contribute to swimming below 80% U_{crit} in cyprinids (Jones, 1982). While sustained swimming has received limited interest compared to other swimming classifications, it can provide important information on bioenergetic and hormonal effects of aerobic exercise on fish (Hammer, 1995). To generate meaningful data, testing sustained swim speed requires prolonged experimental times and a large sample of similarly sized fish with similar nutritional statuses. As a result, quantification of this swim type can be time and resource intensive, which may in part explain why this swimming type has received relatively little interest compared to other types.

Burst swimming is characterized by brief periods of anaerobic swimming that is only maintainable for short periods of time (approximately 15-120 seconds; Jones, 1982; Hammer, 1995; Domenici and Blake, 1997). The peak velocity obtained during the burst is then considered the maximum burst swim speed (U_{max}). Burst speed declines exponentially with time, generally decreasing due to exhaustion of intracellular energy stores or accumulated waste products that inhibit muscle locomotion (Brett, 1964; Jones, 1982). This swimming type is typically measured by maintaining fish at zero or low water speed, and then applying an external stimulus to elicit an escape response through rapid acceleration (reviewed by Domenici and Blake, 1997). Therefore, in some species burst performance is of particular interest as it could ecologically relate to escape speed, which could be indicative of the wild organism's ability to evade predators or obtain superior food sources.

Prolonged swimming is a general term that encompasses swimming ability between burst and sustained levels that generally lasts between 2 and 200 minutes and ends with exhaustion (Hammer, 1995). This swimming category has received much attention as a measure of fish swimming ability as it involves elements of both burst and sustained swimming (Brett, 1964; Beamish, 1978; Jones, 1982; Hammer, 1995). Two protocol methods have been derived in order to assess swimming performance within this intensity level: fixed velocity tests and incremental velocity tests. Both tests are designed to measure submaximal, primarily aerobic swimming capacity in fish, but ultimately end with some degree of anaerobic activity yielding organismal fatigue. In fixed velocity tests fish are exposed to a single flow rate that progressively increases until the single (final) test speed is attained (Hammer, 1995). Time until fish fatigue at the test velocity is then calculated as the final endpoint. These tests require high *n* numbers of similarly sized and conditioned fish for statistical purposes, alongside a relatively long experimental

period (Hammer, 1995). As a result, incremental velocity tests are more commonly used to evaluate fish swimming ability. In these tests fish swim against a set velocity that increases in timed steps until exhaustion (reviewed by Beamish, 1978; Hammer, 1995). The time and velocity at which fatigue occurs are used to calculate the Critical Swimming Speed (U_{crit}; Brett, 1964), which is calculated as:

$$U_{crit} = V_p + ((t_f/t_i) \times V_i)$$

Where:

V_p is the final (peak/penultimate) velocity step swam (cm/s)

V_i is the velocity increment (cm/s)

t_f is the time from the last velocity step increase to fatigue (s)

t_i is the time between velocity increase steps (s)

Because body length can influence swim speed (Bainbridge, 1958; Beamish, 1978; Hammer, 1995), size differences in U_{crit} tests should be accounted for by standardizing swimming speeds according to body length (BL). Kieffer (2000) highlighted three limiting factors of anaerobic swimming: 1) levels of anaerobic fuel stores, 2) accumulation of metabolic end-products, and 3) rate of recovery. It follows that exhaustion during U_{crit} testing is probably the result of one or more of these factors.

Despite ongoing studies utilizing U_{crit} tests, standard procedures and protocols have not been established. A wide range of values have been employed for virtually all variables of this test protocol, including velocity increment duration, velocity increase, and acclimation period within the swim chamber (Beamish, 1978; Hammer, 1995; Plaut, 2001). While standard methods are emerging for salmonids, researchers have failed to agree upon a universal U_{crit} protocol, making comparisons between studies (or between species) difficult.

Critical swimming speed evaluates overall swimming ability taking into account aerobic and anaerobic swimming. However, some authors suggest U_{crit} may be less informative regarding wild fish swimming and physiology compared to other measures (*e.g*. burst capacity, or optimal swimming speed, U_{opt}, in which the optimal aerobic swim speed is balanced by minimal energetic costs; Hammer, 1995; McDonald et al., 1998; Tudorache et al., 2008b), but a direct link between these endpoitns and fitness has not been established. Regardless, swimming performance analysis provides important information supplementary to traditional biochemical, morphological, and toxicological endpoints. Critical swimming speed is an organism-level reflection of the functioning of complex physiological and biochemical processes. It could therefore provide more ecologically relevant evaluations of contaminant toxicity than most acute, and some sublethal, endpoints when tested simultaneously with metabolic endpoints (Kolok, 2001). Importantly, as a whole organism endpoint, U_{crit} can be influenced by and reflective of both individual and environmental variability.

1.2.2 Key factors influencing U_{crit}

Swimming performance can be influenced by various environmental and physiological factors. Addressing all factors is beyond the scope of this discussion (for an in depth review, see Hammer, 1995), but a few key biotic and abiotic factors will be discussed here.

Foremost, U_{crit} varies with species, as behavioural, physiological, biochemical elements unique to each species influences swimming. For example, salmonids commonly incur long migratory distances over their lifetime, making this group an excellent candidate for swimming performance studies. As a result salmonids have been studied extensively in swimming performance tests and information derived from this group forms much of the foundation of current knowledge of aerobic swimming in teleosts. Skipjack tuna (*Katsuwonus pelamis*),

alternatively, exhibit specialized ability to achieve very high burst speeds up to 20 BL/s for a short period of time (which is accompanied by high rates of lactate clearance post-exercise; Arthur et al., 1992). Clearly physiological differences between salmonids and tuna could significantly affect swimming performance if the two groups are directly compared. Similarly, Tudorache et al. (2008b) observed fish species with schooling or long distance migratory behaviour (european perch *Perca fluviatilis*, brown trout *Salmo trutta*, roach *Rutilus rutilus*) had higher U_{crit} compared to bottom-dwelling or short-distance migratory species (stone loach *Barbatula barbatula*, bullhead loach *Cottus gobio*) that generally exhibited lower U_{crit}. Differences in U_{crit} can also exist within different populations of the same species. For example, alterations in swimming performance have been reported between subspecies of salmon (Taylor and Foote, 1991; Lee et al., 2003) and three-spine stickleback (Taylor and McPhail, 1986). In most cases, U_{crit} variation within species can be attributed to geographical differences within populations, and the associated size- and trait-dependent selection in different environments.

Oxygen availability is another principal determinant of U_{crit}. During U_{crit} tests at least 75% of swimming is expected to be aerobic (Webb, 1971a). Therefore, reduced oxygen availability to aerobic tissues (whether due to environmental hypoxia or physiological inability) could significantly affect U_{crit}. For example, Farrell's review (2007) on cardiorespiratory function highlights the importance of adequate physiological oxygen delivery to tissues during swimming. Alternatively, it is well established that environmental hypoxia leads to reduced U_{crit} in various species (Jones, 1971; Bushnell et al., 1984; Petersen and Gamperl, 2010).

As fish are poikolothermic, temperature strongly influences physiological functions, including swimming performance (Myrick and Cech, 2000; Herbing, 2002; Farrell, 2007). Studies investigating the effect of temperature on U_{crit} and/or cardiac performance typically

observe peak activity at optimal physiological temperature that decreases at temperatures above or below this point (Brett, 1964; Myrick and Cech, 2000; Lee et al., 2003; Sandblom and Axelsson, 2007). While reduced U_{crit} at suboptimal temperatures could be due to temperature-related physiological impairments, water temperature also influences oxygen solubility. Therefore, increased temperature can reduce oxygen availability, which could have contributed to cardiorespiratory failure observed in fish swimming at higher temperatures in previous studies (Lee et al., 2003; Farrell et al., 2008). Furthermore, salmonids can encounter highly variable temperature and oxygen availability during migration. In one study it was demonstrated that fatigue at high temperatures was ultimately the result of the failure to increase cardiac output with temperature, rather than reduced oxygen delivery to the gills or gill oxygen diffusion (Steinhausen et al., 2008).

1.2.3 Swim motion

A significant proportion of propulsive movement during swimming depends on caudal fin and whole body motion (Webb, 1975). Swim motion classifications are based on these propulsive movement patterns. For example, most long, thin fish are considered anguilliform swimmers, generating forward thrust through whole-body undulation, creating a full wave cycle over the entire body length. Fish larvae are also often classified with this form of swimming, but transition to other forms as growth progresses. Alternatively, short, stocky fish like tuna are considered thunniform swimmers and rely predominantly on caudal movement for propulsion. Salmonids and cyprinids are subcarangiform swimmers (Figure 1.3A; Webb, 1975; Lindsey, 1978; Kaufmann, 1990). This movement is characterized by body and tail oscillation along the lateral axis as the body encompasses one half to one whole wavelength. Excepting anterior body centralization along the midline, subcarangiform movement is generally comparable to

anguilliform swimming (Webb, 1975). Variation in swim motion can be characterized by recording movements in a swim chamber during a swimming performance test and comparing body form at different periods within the swim cycle.

Different endpoints exist to characterize swim motion. Tail beat frequency (the number of beat cycles the fish tail completes over a given period of time) is a relatively uncomplicated, easily obtainable indicator of locomotor activity in both laboratory and field studies. A close positive relationship between swimming speed and tail beat frequency has long been established in carangiform and subcarangiform swimmers (Bainbridge, 1958; Ohlberger et al., 2007). Therefore, this endpoint could provide important supplementary information when assessing U_{crit} in different fish. Furthermore, tail beat frequency can be used as a predictive tool for U_{crit} and oxygen consumption during steady swimming in some fish (Steinhausen et al., 2005). Tail beat amplitude can also be considered when characterizing swim motion (Figure 1.3B). This endpoint is based on the lateral movement of the fish tail as it deviates left and right from the midline during steady swimming. As a result, this endpoint can be useful in providing information on the muscle power complementing tail beat frequency (Herbing, 2002; Tudorache et al., 2010).

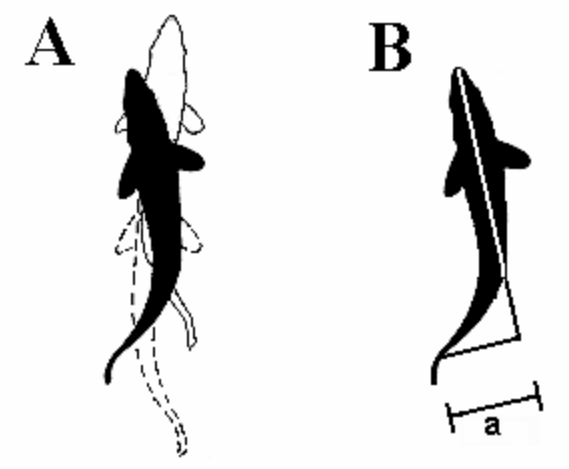

Figure 1.3 Dorsal view of subcarangiform swim motion positions. (A) Black silhouette superimposed on successive swim motion positions through one tail beat cycle (positioned one-half tail beat earlier and one-half tail beat later). (B) Tail beat amplitude (a) image analysis during subcarangiform swimming, measured as the distance from the midline to left- or right-most point of the furthest posterior point of the caudal peduncle through one tail beat cycle. Images modified from Lindsey (1978), used with permission from Elsevier Books.

1.3 Physiology and metabolism of swimming

Swimming integrates many different, complex systems within an organism. In this section the basic physiological and biochemical elements involved in aerobic and anaerobic swimming are addressed.

1.3.1 Muscle types

It is estimated total muscle mass accounts for approximately 60-85% of total body mass in teleosts (Moyes and West, 1995; Hochachka and Somero, 2002). In fish, red and white muscle fibres are anatomically separated, which allows for clear characterization of fibre type based on aerobic capacities. Red (slow-twitch) muscle generally accounts for 5-15% of total muscle mass (Moyes and West, 1995). Most red muscle fibres are arranged in longitudinal bands along the lateral midline on each side of the fish and are important for slower, sustained movements, such as those employed during migratory periods or foraging (Gibb and Dickson, 2002). Red muscle has a high aerobic capacity and is characterized by high levels of vascularisation and relatively high numbers of intracellular mitochondria (Moyes et al., 1992). As a result, this muscle type is used for aerobic activity, and is relied upon for swimming at or below 80% U_{crit}. Intramuscular lipids are the primary fuel substrate oxidized during activity (Moyes and West, 1995), but carbohydrates could also play some role in fuelling aerobic activity, possibly accounting for up to 10% of oxidized substrates at maximal aerobic capacity (West et al., 1993).

White (fast-twitch) muscle accounts for approximately 85-95% of total muscle mass in most teleosts (Moyes and West, 1995; Coughlin, 2002). This fibre type provides power for short, anaerobic bursts of high swim speeds and accelerations, relying initially on available intramuscular substrates (free ATP), followed by intramuscular glycogenolysis. Low oxygen dependence in white muscle is supported by observations of substantially less vascularisation

and mitochondrial numbers compared to red muscle (West et al., 1993). In swimming performance tests it is estimated white muscle is recruited at swim speeds near or beyond 80% U_{crit}, as red muscle oxygen becomes limiting (Webb, 1971a). It is also highly plausible white muscle makes a small contribution to aerobic swimming, but the degree to which this occurs is unclear (Jones, 1982; Moyes et al., 1992; Rajotte and Couture, 2002).

1.3.2 Specific intermediate metabolic fuels catabolised during swimming

Glycogen and triglycerides are the main forms of stored energy used during activity, but energy molecule preference can vary with species and tissue metabolic capacity (Tullis et al., 1991; Moyes et al., 1992; Dickson, 1995). It follows that the swimming and muscle types most commonly employed during routine activity will ultimately dictate organism dependence on one form of stored energy over another.

Stored lipids play key roles in growth and survival in young fish (Pratt and Fox, 2002; Biro et al., 2004), and triglycerides account for the vast majority of stored acylglycerols, the major storage form of lipids (Henderson and Tocher, 1987; Jobling, 1994). While carbohydrates and proteins may be used during activity, lipids are the primary fuel source during aerobic swimming, and are thus primarily oxidized by red muscle. This observation is further supported by large mitochondrial oxidative capacity in red muscle of more active species (Moyes et al., 1992; Moyes and West, 1995). Triglycerides are primarily stored in and mobilized from liver, muscle and visceral tissue, and transported in the circulation as free fatty acids. However, it is unclear which stores are preferentially mobilized during activity and why (reviewed by Moyes and West, 1995; van den Thillart et al., 2002).

Glycogen is the main form of stored carbohydrate (Jobling, 1994). In fish glycogen is primarily stored in liver and to a lesser degree muscle tissue. Although glycogen and glucose

play central roles in mammalian metabolism, their utility and function seem to be diminished in fish (Hemre et al., 2002). Hepatic stores can play an important role in fish acute stress response (discussed below). Alternatively, large glycogen stores have been observed in some coldwater species, which may increase hypoxia tolerance during periods of ice-cover (Jobling, 1994). Overall, glycogen in fish plays a key role in burst activity and anaerobic swimming, the success of which is dependent on available intramuscular stores (Moyes and West, 1995). While the liver is a key energy molecule storage site, evidence suggests the role of hepatic glycogen as a glucose source during anaerobic swimming is fairly minimal (Milligan and Wood, 1987). It is plausible hepatic glycogen stores play some role during strenuous exercise, but given the total body volume occupied by white muscle and its lack of vascularisation, most anaerobic activity is probably fuelled by intramuscular glycogenolysis (Moyes and West, 1995).

While there is near-continuous whole body protein turnover in most fish, exercise-related protein catabolism can also provide some fuel for swimming activity (Jobling, 1994). In one study, protein catabolism contributed to less that 30% of total fuel used in laboratory raised rainbow trout (*Oncorhynchus mykiss*; withheld 3 days from feeding) swimming 75% U_{crit} at 15°C (Kieffer et al., 1998). However, at 5°C (withheld 5 days from feeding), protein catabolic contribution to swimming fell to approximately 15% (Kieffer et al., 1998). Conversely, salmonids preferentially metabolize lipids during migratory runs, generally metabolizing protein only after lipid stores are exhausted (Mommsen et al., 1999). Overall, protein catabolism probably contributes relatively little to swimming metabolism in non-migratory fed fish until carbohydrate and lipid stores become unavailable (Moyes and West, 1995).

Freely circulating glucose could be utilized by anaerobic glycolytic tissues (white muscle) during exhaustive swimming, but contribution as a fuel source is likely minimal. As

discussed above, poor vascularisation suggests circulatory contribution of fuel substrates to white muscle metabolism is likely quite low. For example, in trout swimming 80% U_{crit}, West et al. (1993) observed no change in white muscle utilization of free glucose, while Moyes and West (1995) suggest in most species plasma levels are probably inadequate to supply energy needed for burst exercise, except at lowest intensities. Interestingly, trout swimming 80% U_{crit} have been shown to increase red muscle glucose utilization (from the circulation) 28-fold (West et al., 1993). However, the authors calculated red muscle glucose oxidation accounted for only 5-10% total substrates metabolized during swimming (West et al., 1993).

Lactate generated during anaerobic swimming can also be recovered during activity and used as a fuel substrate through its conversion to pyruvate. However, it appears the utility of lactate as a metabolic substrate during activity is also minimal. This is probably again due to the fact that high muscular demand for oxidizable substrates generally exceeds circulatory supply (Moyes and West, 1995). Instead, the majority of intramuscular and circulatory lactate generated during anaerobic swimming is likely recovered post-exercise as glycogen. The recovery process often requires 4 to 12 hours before returning to normal pre-exercise levels, further suggesting lactate clearance during anaerobic activity contributes minimally to anaerobic ATP generation (reviewed by Kieffer, 2000).

1.3.3 Biochemical pathways utilized during swimming

As discussed above, lipids, carbohydrates and protein can all be metabolised during swimming. While these macromolecules are catabolised through vastly different pathways, final metabolites eventually feed into similar pathways yielding oxidative phosphorylation, ultimately generating ATP. For example, aerobic catabolism of glycogen or triglycerides eventually generates acetyl coenzyme A, which can directly enter the citric acid cycle. Similarly, aerobic

protein catabolism can also yield acetyl coenzyme A, but this process may also include complex, intermediate steps that generate various metabolites that enter different parts of the citric acid cycle (Jobling, 1994). However, because U_{crit} ultimately incorporates both aerobic and anaerobic swimming, intracellular ATP will be derived from specific metabolic pathways depending on activity level. Key biochemical pathways utilized during U_{crit} tests and important markers of these pathways are briefly discussed here.

1.3.3.1 Fatty acid β-oxidation spiral

Fatty acid β-oxidation is the process through which triglycerides are aerobically catabolised into useful metabolites to enter the citric acid cycle (Jobling, 1994). In this process fatty acids from triglycerides pass through a series of steps that results in one acetyl coenzyme A molecule and a fatty acid ester with two fewer carbon atoms than the original (Figure 1.4). The fatty acid ester can then re-enter the β-oxidation spiral thus reducing the chain by two more carbons and generating another acetyl coenzyme A molecule. A rate-liming step in fatty acid β-oxidation involves the enzyme β–hydroxyacyl coenzyme A dehydrogenase (HOAD). Elevated HOAD activity is characteristic of tissues with greater aerobic capacity and lipid metabolism, exemplified by red muscle, which is recruited during sustained swimming (Moyes et al., 1992; Dickson, 1995). In some studies the activity of carnitine palmytoyltransferase (CPT), which facilitates the transport of long chain fatty acids across membranes, can also be used as an indicator of tissue ability to access fatty acids and mitochondrial function. For example, comparing tuna and carp (*Cyprinus carpio*) muscle tissues, Moyes et al. (1992) observed that CPT activity increases with tissue aerobic capacity. However, testing HOAD tissue activity has become a relatively common assay (at least in studies investigating fish tissue metabolic activity) for

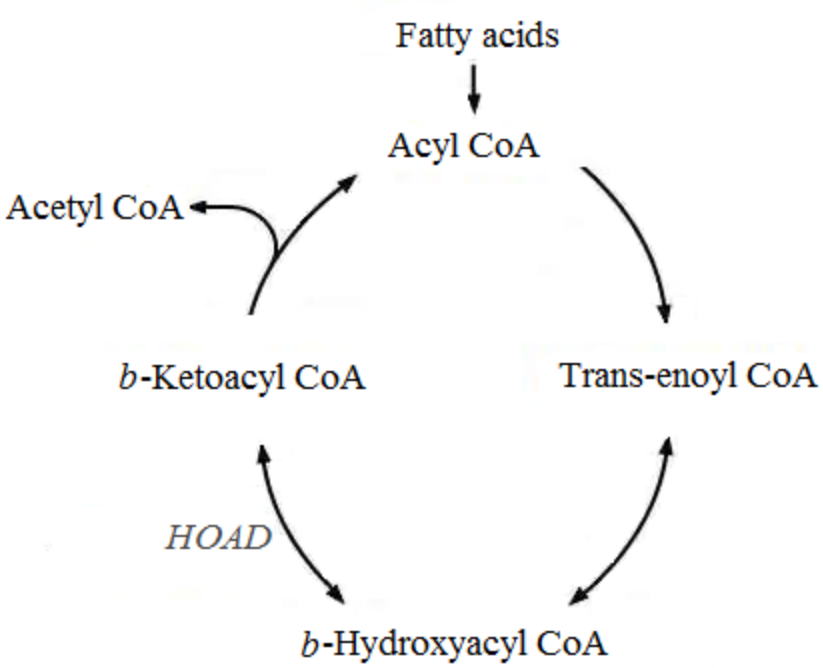

Figure 1.4 Summary diagram of fatty acid β-oxidation cycle within the mitochondrial matrix. CoA, coenzyme A; HOAD, β-hydroxyacyl coenzyme A dehydrogenase.

assessing aerobic lipolytic capacity (Pelletier et al., 1994; Londraville and Duvall, 2002; Rajotte and Couture, 2002).

1.3.3.2 The citric acid cycle

The citric acid cycle involves a series of enzyme-catalyzed reactions, the by-products of which feed into the electron transport chain. The citric acid cycle is the key mechanism for ATP production under aerobic conditions and generates substantially more ATP than anaerobic pathways (*e.g.* anaerobic glycolysis, discussed below). A full review on the citric acid cycle is beyond the scope of this discussion, but the pathways involved are briefly summarized here (Figure 1.5). It is important to note that all cycle steps are enzyme-catalyzed but for clarity only citrate synthase (CS) is shown on Figure 1.5.

Acetyl coenzyme A derived from fatty acid β-oxidation and aerobic glycolysis (the breakdown of glucose to pyruvate) feeds into the citric acid cycle. The conversion of acetyl coenzyme A and oxaloacetate to citrate is generally viewed as the initial step of the citric acid cycle, which is facilitated by CS, a rate-limiting enzyme. As an important regulatory step for the citric acid cycle, tissue CS activity is considered indicative of aerobic capacity (Dickson, 1995; Rajotte and Couture, 2002; Lemos et al., 2003). Elevated CS activity is therefore observed in tissues that efficiently generate aerobic energy (Moyes et al., 1992; Dickson, 1995) and could be useful in the determination of aerobic scope and U_{crit} performance.

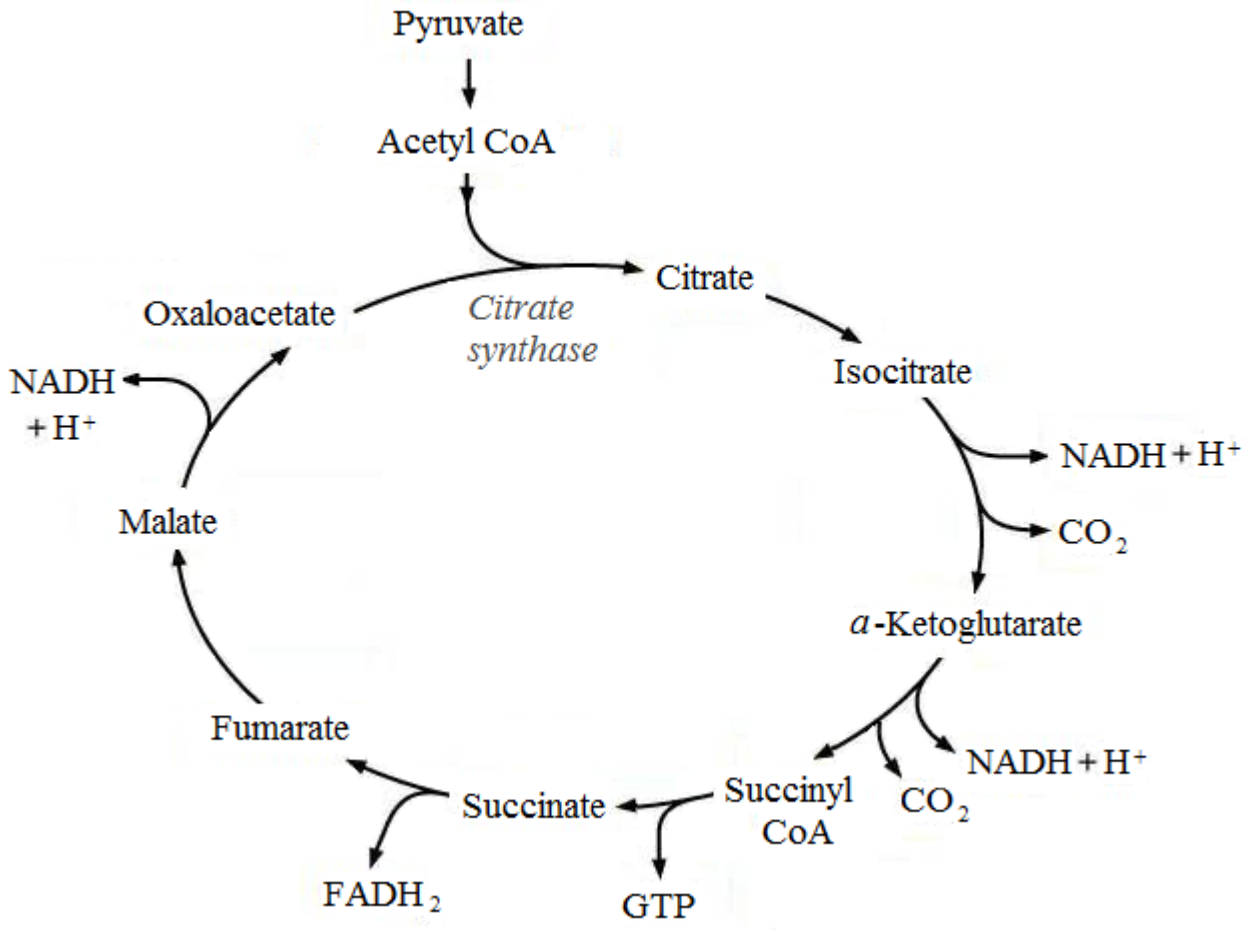

Figure 1.5 Summary diagram of citric acid cycle within the mitochondrial matrix. CoA, coenzyme A; CO_2, carbon dioxide; $FADH_2$, flavin adenine dinucleotide; GTP, guanosine triphosphate; NADH, nicotinamide-adenine dinucleotide. Copyright © 2004 From Essential Cell Biology, 2nd edition by Bruce Alberts, et al. Reproduced by permission of Garland Science/Taylor & Francis Books, Inc.

1.3.3.3 Anaerobic glycolysis

During anaerobic glycolysis, glycogen is catabolised into individual glucose monomers through the same mechanisms utilized under aerobic conditions (glycogenolysis). Each glucose molecule yields two molecules of pyruvate. During aerobic metabolism pyruvate is then catabolised to acetyl coenzyme A, which enters the citric acid cycle. However, during anaerobic glycolysis pyruvate is instead converted to lactate by lactate dehydrogenase. This process generates considerably less ATP than aerobic metabolism and activity fuelled through anaerobic mechanisms is limited in duration. Upon exhaustive swimming, lactate builds up in white muscle and enters the systemic circulation, which can lower tissue and circulatory pH (Jobling, 1994; Moyes and West, 1995; McDonald et al., 1998). As a result, measuring blood lactate can be used to evaluate an individual's ability to anaerobically utilize intramuscular glycogen reserves (Rajotte and Couture, 2002).

1.3.4 Cardiovascular elements of exercise

The cardiovascular system plays a substantial role during swimming and can be a key determinant of U_{crit} ability. Oxygen uptake and distribution to aerobic tissues during swimming is dependent on a functional cardiovascular system. Impairment to this system could lead to early onset of anaerobic metabolism during U_{crit} tests, leading to earlier fatigue. At or approaching U_{crit} the cardiorespiratory system is likely operating at maximal capacity (Farrell, 2007), which coincides with maximal oxygen consumption. Furthermore, cardiac output generally increases with swimming speed. Rainbow trout with higher U_{crit} are reported to be capable of greater cardiac output compared to fish with lower U_{crit} (Claireaux et al., 2005). Interestingly, in the same study poor swimming fish had a morphologically different heart structure than superior

swimmers, which suggests subtle cardiovascular morphological and performance changes could significantly affect swimming ability (Claireaux et al., 2005).

Hematocrit is the volume of blood occupied by red blood cells. During an acute stress response or an elevation in activity level, splenic release of erythrocytes can increase oxygen-carrying capacity in order to meet metabolic demands. The ability to increase hematocrit with increasing levels of activity could play an important role in fuelling aerobic metabolism and swimming performance. Correspondingly, hematocrit has been observed to increase with U_{crit} in some salmonids (Butler et al., 1992; Gallaugher et al., 1995), but not others (Thorarensen et al., 1993; Steinhausen et al., 2008). Hematocrit can also increase in response to stress including experimental stressors, such a handling stress, water quality (Butler et al., 1992) or temperature fluctuations (Sandblom and Axelsson, 2007).

1.3.5 Stress response

Acute stress response pathways are closely tied to those involved in exercise and energy homeostasis and therefore could be relevant when examining intermediary metabolism and swimming performance. Physiological and biochemical coping mechanisms to environmental stressors can be categorized into primary, secondary and tertiary responses. Upon exposure to an acute stress stimulus, the primary stress response is initiated by neuroendocrine pathways (Jobling, 1994). Upon stimulation, the chromaffin tissue releases catecholamines which enter systemic circulation. Likewise, adrenocorticotropic hormone (ACTH) secreted from the corticotropes enters the blood stream, stimulating the release of corticosteroids from the head kidney. Cortisol is the primary corticosteroid hormone in teleosts (Jobling, 1994). Compared to catecholamines, systemic cortisol secretion is slower, but the duration of its effects are longer

(Martinez-Porchas et al., 2009). Upon entry into the system, these stress hormones can trigger a range of secondary metabolic and cardiovascular effects.

The secondary stress response encompasses rapid blood and tissue changes to mobilize energy reserves and assist the organism in mediating the stress response (Jobling, 1994). Catecholamines are involved in modulating cardiovascular and respiratory function to ensure sufficient oxygen is delivered to tissues and to mobilize energy stores by increasing liver glycogenolysis, liver gluconeogenesis and lipid mobilization from a variety of tissue stores (reviewed by Reid et al., 1998). Systemic cortisol targets the liver, stimulating hepatic glycogen mobilization, followed by elevated circulatory glucose. Consequently, splenic contraction increases hematocrit to elevate blood oxygen-carrying capacity, while plasma lactate can increase if the fish engages anaerobic glycolytic pathways as a physical response to the stressor. These endpoints can be used as indicators of secondary physiological stress. For example, elevated plasma lactate, glucose and hematocrit were observed in migrating adult sockeye salmon that experienced higher migratory temperatures than counterparts that experienced lower temperatures (Mathes et al., 2010).

After a single stress stimulus, baseline biochemical and physiological changes return to pre-exposure levels in a matter of hours to days. Upon chronic exposure however, pathological changes can be observed, which can be considered part of a tertiary stress response. Chronic stress can ultimately lead to decreases in reproductive stress, growth, and pathogen immunity (Jobling, 1994).

1.4 Effects of contaminant exposure on swimming and energy homeostasis

1.4.1 Contaminant exposure and swimming performance

Reduced swimming performance due to contaminant exposure could negatively affect individual survival and population sustainability, even in the absence of overt organismal toxicity based on traditional toxicity endpoints (LC_{50}s, EC_{50}s, reproductive effects; Scott and Sloman, 2004; McKenzie et al., 2007). Therefore, not only could U_{crit} performance be a sensitive indicator of contaminant stress and toxicity, but it is also considered an ecologically relevant assessment of swimming ability (reviewed by Plaut, 2001). Critical swimming speed tests can provide important information on organism-level effects of contaminant exposure when considered in addition to morphological and biochemical endpoints, providing close correlations to fitness and survival (Plaut, 2001). Indeed, a number of contaminants have been shown to negatively affect U_{crit}, including ammonia (reviewed by McKenzie et al., 2003) and dissolved metals (Wilson and Wood, 1992; Beaumont et al., 1995; Alsop et al., 1999; McGeer et al., 2000; Rajotte and Couture, 2002; Taylor et al., 2004) as well as complex mixtures such as crude oil fractions (Kennedy and Farrell, 2006), and urban river systems (McKenzie et al., 2007).

Treated Key Lake uranium mill effluent contains a number of contaminants of interest that could negatively affect swimming in downstream fish. Therefore, a number of studies reporting negative effects of complex metal mixtures on U_{crit} in wild fish are of particular interest. Hopkins et al. (2003) collected juvenile lake chubsuckers (*Erimyzon sucetta*) from an uncontaminated bay and exposed fish 90-100 days to metal-laden ash-contaminated sediment. Exposed fish accumulated higher concentrations of selenium, vanadium, strontium, and arsenic, while U_{crit} in exposed fish was half that of control fish (Hopkins et al., 2003). In other studies, environmental metal contamination significantly altered tissue metabolic capacity in wild yellow

perch (*Perca flavescens*), but effects on U_{crit} were variable (Rajotte and Couture, 2002; Audet and Couture, 2003; Taylor et al., 2004). Alternatively, laboratory studies have shown that metals such as aluminum and copper can directly affect gill or haematological capacity, which can also significantly impair U_{crit} (Wilson and Wood, 1992; Beaumont et al., 1995; McGeer et al., 2000).

Ammonia is used throughout the uranium ore milling process and can be discharged at relatively high concentrations for short periods of time. This contaminant has been shown to negatively affect swimming performance in fish, possibly causing premature white muscle fatigue via partial depolarisation of muscle fibre membrane (Randall and Tsui, 2002; McKenzie et al., 2003). Therefore, ammonia could negatively affect swimming performance in fish downstream of the Key Lake uranium mill during periods of burst swimming or at approximately 80% U_{crit} as white muscle fibres are recruited. This hypothesis is supported by observations of reduced tail beat frequency and tail amplitude in laboratory raised fish exposed to ammonia (Shingles et al., 2001; Tudorache et al., 2010).

1.4.2 Contaminant exposure and intermediary metabolic effects

Contaminant exposure in low water temperatures can stimulate additional energy expenditure beyond basic metabolic demands, which could sufficiently deplete energy stores in wild fish to impact survival (Lemly, 1996). Lemly (1993) described these overwintering effects of contaminant exposure (in warm-water fish) as Winter Stress Syndrome (WSS). In this previous study decreased oxygen consumption, body condition factor, total body lipids, and increased cumulative mortality were observed in warm-water bluegill sunfish (*Lepomis macrochirus*) exposed to elevated selenium in winter conditions (Lemly, 1993). The degree to which WSS occurs in cold-water fish in the presence of uranium milling effluent is questionable. Based on the WSS hypothesis, decreased condition factor, energy stores, and overwinter

survivability should be observed in northern fish exposed to uranium milling effluent. However, elevated energy stores in juvenile and adult fish downstream of the Key Lake uranium mill suggests these fish are relatively healthy compared to their unexposed counterparts (Bennett and Janz, 2007; Kelly and Janz, 2008). This assumption is based on the observation that whole body lipid stores are positively correlated with survival and growth (Post and Parkinson, 2001). However, elevated energy stores in fish downstream of the mill could be the result of a number of factors related to uranium milling effluent exposure. While many of these factors are beyond the scope of this discussion, a few key possibilities are summarized below.

Foremost, elevated energy stores in fish downstream of the Key Lake uranium mill could be attributed increased food web productivity in exposure lakes due to elevated ammonia in milling effluent (Muscatello et al., 2006; Bennett and Janz, 2007). As northern boreal aquatic systems are generally quite low in nutrients, a nutrient influx (of nitrogen in the form of ammonia) could increase system productivity as normally limiting nutrients become widely available (Elser et al., 1990). This could contribute to elevated energy stores in higher trophic-level organisms. Indeed, some condition patterns in small bodied fish (increased size, size-at-age, condition factor) suggest nutrient enrichment has occurred in Delta Lake with enhanced availability of food resources (Golder Associates, 2008). However, Kelly and Janz (2008) suggest elevated energy stores in upper trophic level fish were not linked to food web enrichment (prey triglyceride concentration, fish stomach content wet weight). Alternatively, the high ion contribution from discharged Key Lake uranium milling effluent could contribute to a more homeostatic environment for fish ionoregulation. In addition to nutrient limitation, boreal water bodies are characterized by very low ionic strength (*i.e*. soft water). Environmental conductivity closer to physiological levels could allow fish to allocate energy towards greater stores or

growth, rather than expending energy towards osmoregulation (Shuter et al., 1989). Indeed, greater condition factor has been associated with elevated water conductivity in some freshwater species (Dennis and Bulger, 1995; DiCenzo et al., 1996), but not others (Copp, 2003). Obviously mill effluent directly contributes to elevated water hardness and conductivity of downstream lakes, but the effects on energy stores require investigation.

Alternatively, effluent exposure could biochemically affect the ability of fish downstream of the mill to adequately utilize energy reserves compared to fish from reference lakes. Recent studies in wild perch suggest complex metal exposure alters tissue metabolic capacity at the biochemical level, interfering with energy storage and usage pathways (Levesque et al., 2002; Rajotte and Couture, 2002; Audet and Couture, 2003). It is plausible some effluent components inhibit key lipolytic or glycolytic pathways, substantially limiting energy reserve mobilization or catabolism during activity. Therefore, it is unclear how uranium milling effluent exposed fish utilize energy reserves, and why these reserves do not diminish in wild fish in the presence of environmental stressors as expected. If metabolic alterations are present, this could have significant effects on swimming performance.

1.4.3 Selenium toxicity

Discharged treated mill effluent from the Key Lake operation contains elevated selenium, which is a central contaminant of interest in lakes downstream of the uranium mill. Selenium characterization in downstream water bodies has been the subject of numerous investigations at the Key Lake operation (Muscatello et al., 2006; Robertson and Liber, 2007; Muscatello et al., 2008; Wiramanaden et al., 2010) and could have significant effects on swimming ability in resident fish in the present study. This metalloid is of particular interest as many fish species are highly sensitive to elevated environmental selenium, and early life-stage organisms are

particularly vulnerable. Toxicity is primarily based on the shared chemical characteristics of selenium and sulfur (Palace et al., 2004). Disulfide bonds between amino acids cysteine and methionine are essential for proper protein structure, but during amino acid synthesis cells may indiscriminately substitute selenium in a dose-dependent manner with sulphur. This process generates selenoamino acids (most notably, selenomethionine or Se-met), which form triselenium linkages or selenotrisulfide linkages, instead of disulfide bonds, upon protein assembly (Stadtman, 1974; Lemly, 2002; Palace et al., 2004). Thus, improperly formed proteins fail to respond appropriately to biochemical cues. As a result environmental selenium can interrupt a number of physiological processes required for growth and survival.

Female oviporous vertebrates transfer organic selenium into eggs with high efficiency, with toxicity incurring as embryos metabolize Se-met deposits in yolk (Heinz et al., 1989; Lemly, 2002; Spallholz and Hoffman, 2002; Holm et al., 2005; Muscatello et al., 2006). Palace et al. (2004) suggest rapidly growing organisms (*i.e.* larval and juvenile fish) exhibit heightened sensitivity to selenium due to elevated protein synthesis during periods of rapid growth. Therefore, the greatest overt manifestations of selenium toxicity are often observed in larval and juvenile stages, which subsequently experience high mortality. Adult fish from selenium-contaminated environments can thrive, but reproductive function is often lost due to gonadal and teratogenic effects (Lemly, 2002).

Characteristic developmental abnormalities are well documented in selenium exposed fish, including skeletal curvatures, craniofacial deformities, fin deformities, cardiovascular defects and edema culminating in mortality (Lemly, 2002; Holm et al., 2005; Muscatello et al., 2006). Clearly, these effects have the potential to cause massive larval mortality leading to significant population effects. However, it is unclear if lesser degrees of selenium toxicity exist

in some individuals, which could manifest as sublethal, latent deformities or bioenergetic alterations. While overt lethal pathologies could cause immediate population effects, individuals with sublethal defects, such as subtle morphological terata, could reach adult stages, but overall functional ability within the environment could be impaired. As fish downstream of the Key Lake mill have elevated incidence of larval mortalities, research investigating possible latent deformities in older individuals is warranted (Muscatello et al., 2006). Therefore, when assessing swimming performance it is important to take into account swim motion in fish, as subtle morphological deformities could affect swimming ability. Alternatively, reduced U_{crit} and tail beat amplitude was observed in naïve adult zebra fish (*Danio rerio*) exposed only to dietary Se-met (Thomas and Janz, 2011). It was hypothesized in this previous study that impaired swim motion could be due to the incorporation of Se-met during ongoing protein synthesis that led to the development of dysfunctional proteins in tissue (Thomas and Janz, 2011). This hypothesis could hold negative implications for wild fish in which developmental abnormalities are absent, but have the potential to develop swim motion impairments as they mature in selenium contaminated environments.

Cardiovascular development is of particular interest in fish exposed to Key Lake uranium operation effluent. In addition to previously described morphological deformities, common cardiovascular effects of selenium exposure include pericardial edema, pericarditis, and myocarditis (Lemly, 2002). These cardiovascular abnormalities are of particular interest, especially as they could relate to swimming performance. As discussed above, cardiovascular function is essential to swimming ability, and U_{crit} performance is dependent on cardiorespiratory and vascular function (Butler et al., 1992; Gallaugher et al., 1995; Claireaux et al., 2005; Farrell, 2007). Therefore, effects of latent cardiovascular deformities in fish downstream of the Key

Lake uranium mill, if they exist, are unclear, but could negatively affect swimming performance. Histological evaluation of cardiovascular structures (*e.g*. bulbus arteriosus, ventricle, dorsal aorta width) in effluent exposed fish could provide insight on sublethal morphological effects of uranium milling effluent exposure in juvenile and adult fish.

1.4.4 Contaminant exposure and stress response

There is growing evidence that chronic metal exposure in some fish impairs the stress response. Generally, chronic contaminant exposure can elicit pathological changes (as tertiary stress response effects) that include reduced growth rates and reproductive success (Jobling, 1994). However, some studies report a blunted primary stress response (impaired cortisol production) in yellow perch from metal contaminated lakes subjected to acute stress tests compared to fish from clean lakes (Brodeur et al., 1997; Laflamme et al., 2000; Levesque et al., 2002; Gravel et al., 2005). Gravel et al. (2005) also reported no change in liver glycogen after the same tests in fish from metal contaminated lakes, while fish from clean lakes significantly decreased liver glycogen. Copper has also been shown to impair cortisol secretion at high concentrations through direct adrenocortical cell toxicity (Gagnon et al., 2006). However, the mechanism of action of other metals or metal mixtures on the stress response in fish remains unclear. Because of the close association of stress hormones with energy stores and intermediary metabolism, the investigation of components of the acute stress response in fish downstream of the Key Lake mill in U_{crit} tests is warranted.

1.5 Northern minnow species of interest

Spottail shiner are medium-sized minnow of the Cyprinidae family, native to northern Saskatchewan and widely distributed throughout Canada and parts of the United States (Scott and Crossman, 1998). Shiner are facultative schoolers (Seghers, 1981), and schools of mature

and/or juvenile fish can be observed in many lakes near and downstream of the Key Lake uranium mill. Fish can achieve 5-13 cm fork length at maturity, and are easily identified from other similar-looking species by a characteristic black spot at the base of the caudal peduncle. Time to sexual maturity is unclear, but has been reported in yearling fish with spawning generally occurring in spring or early summer (Scott and Crossman, 1998). Fish tend to forage on plankton, some macrophytic plant material, and some insects. Spottail shiner are an important prey species for higher trophic aquatic organisms, including most predatory fish species, and some piscivorous birds, and Scott and Crossman (1998) describe this species as "one of the most important forage cyprinids in Canadian lakes". Toxicologically, relatively little is known of spottail shiner sensitivity to contaminants or water quality tolerance. While this fish is commonly captured and sold as bait for game fishing, breeding and husbandry operations appear to be unexplored or perhaps unsuccessful. Therefore, shiner cannot be obtained commercially, limiting the ability to perform laboratory experiments with this species.

In contrast, fathead minnow (FHM) are the most widely used small fish model for regulatory ecotoxicology in North America and are commonly used for aquatic toxicology research (Ankley and Villeneuve, 2006). This species, which is also a cyprinid, has a well-defined reproductive/development cycle and generally tolerates a wide range of water chemistry conditions (Ankley and Villeneuve, 2006). In North America, wild FHM can be found across a wide range of lacustrine or riverine bodies, but are absent from lakes near the Key Lake uranium mill. Fish reach maturity approximately 4-5 months after hatching, with adult males developing distinctive secondary sex characteristics. Adult fish generally reach approximately 5 cm fork length. This species can be obtained for both commercial and research purposes, as it is easily maintained and bred in laboratory settings. Like spottail shiner, these fish are opportunistic

omnivores, but are commonly maintained on brine shrimp (genus *Artemia*), blood worms (family *Chironomidae*) or flaked food in laboratory settings. Wide availability and well-established developmental stages make this species an excellent candidate for investigating the swimming and physiological effects of uranium mill effluent exposure.

1.6 Null hypotheses and general research methods

1.6.1 Null hypotheses

Specific null hypotheses of this thesis were:

1) Uranium mill effluent exposure would have no effect on energy homeostasis in wild juvenile spottail shiner collected downstream of the mill and juvenile FHM exposed to effluent in the laboratory.
2) Uranium mill effluent exposure would have no effect on swimming performance in wild juvenile spottail shiner collected downstream of the mill and juvenile FHM exposed to effluent in the laboratory.
3) Uranium mill effluent exposure would have no effect on cardiovascular morphology in wild juvenile spottail shiner collected downstream of the mill and juvenile FHM exposed to effluent in the laboratory.

To assess the effects of Key Lake uranium mill effluent exposure in fish a suite of biochemical, physiological, and morphological endpoints were determined in conjunction with swim motion and swimming performance analysis. Swimming performance was based on Brett's (1964) critical swimming speed model. Tissue triglyceride and glycogen levels were measured in fatigued and non-fatigued fish to evaluate energy stores and mobilization during swimming. Plasma lactate, glucose, and hematocrit were also measured in fatigued and non-fatigued shiner. Prior to testing tissue activity in effluent exposed fish, it was necessary to establish assay

methods for CS and HOAD. Once established, CS activity was evaluated in shiner muscle and FHM whole body homogenates to evaluate aerobic ATP generation, while HOAD activity was determined to evaluate lipid utilization.

1.6.2 General methods

In order to test the above null hypotheses, the general methods employed in this thesis were designed to:

1) Establish and validate methods for measuring key rate-limiting enzymes in aerobic energy catabolism (CS and HOAD activity) in shiner muscle and FHM whole body homogenates.
2) Compare swimming performance, energy homeostasis and cardiovascular structures in juvenile spottail shiner collected *in situ* from a uranium milling effluent receiving lake (Delta Lake) and a reference lake (Yeoung Lake; Figure 1.6).
3) Compare swimming performance, energy homeostasis and cardiovascular structures in juvenile FHM bred and reared *ex situ* in control dechlorinated municipal water (Control treatment) or 5% uranium milling effluent/95% dechlorinated municipal water mixture (5% effluent treatment; Figure 1.7).

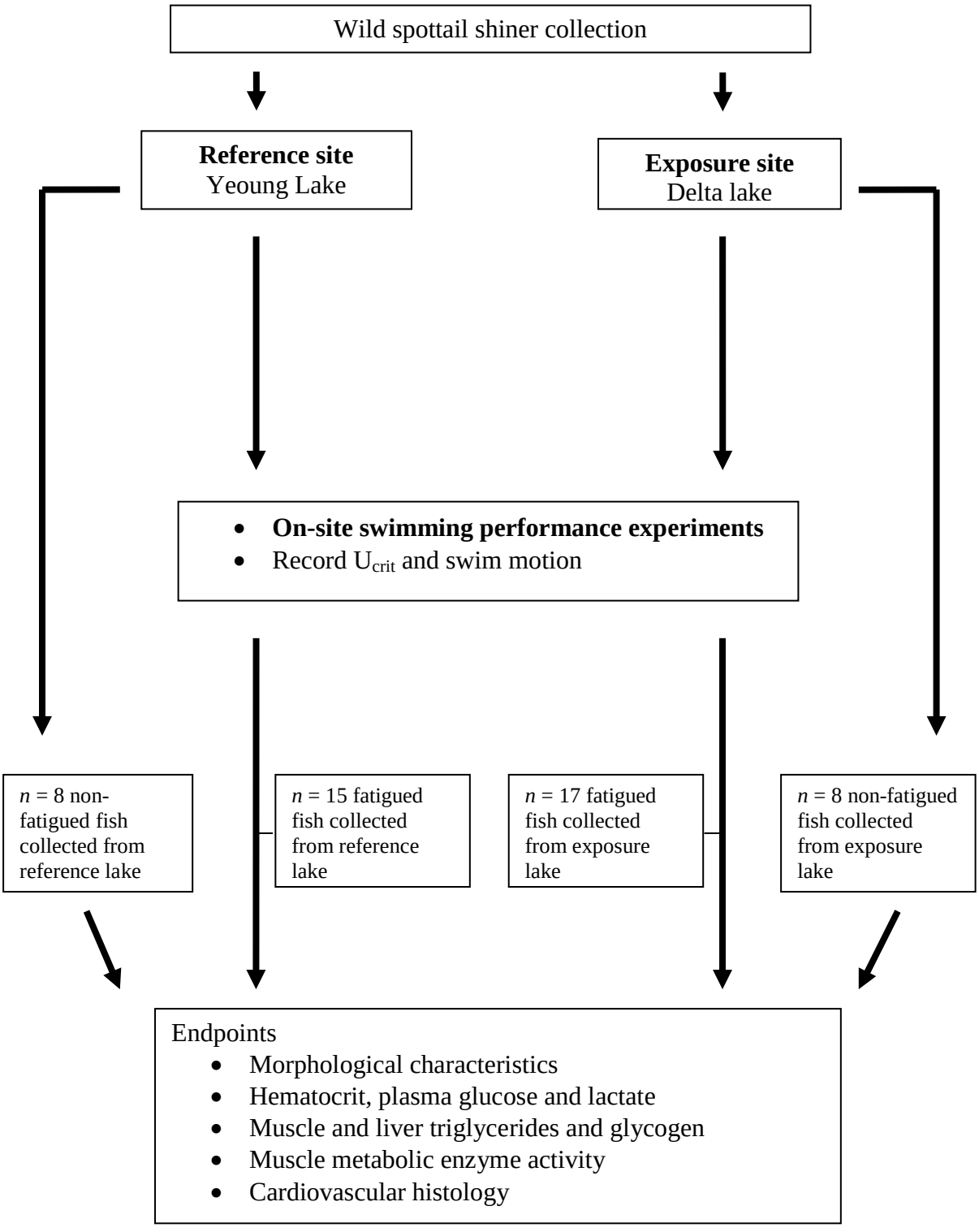

Figure 1.6 Flow chart of field project experimental design for June 2009, Key Lake uranium mill, Saskatchewan.

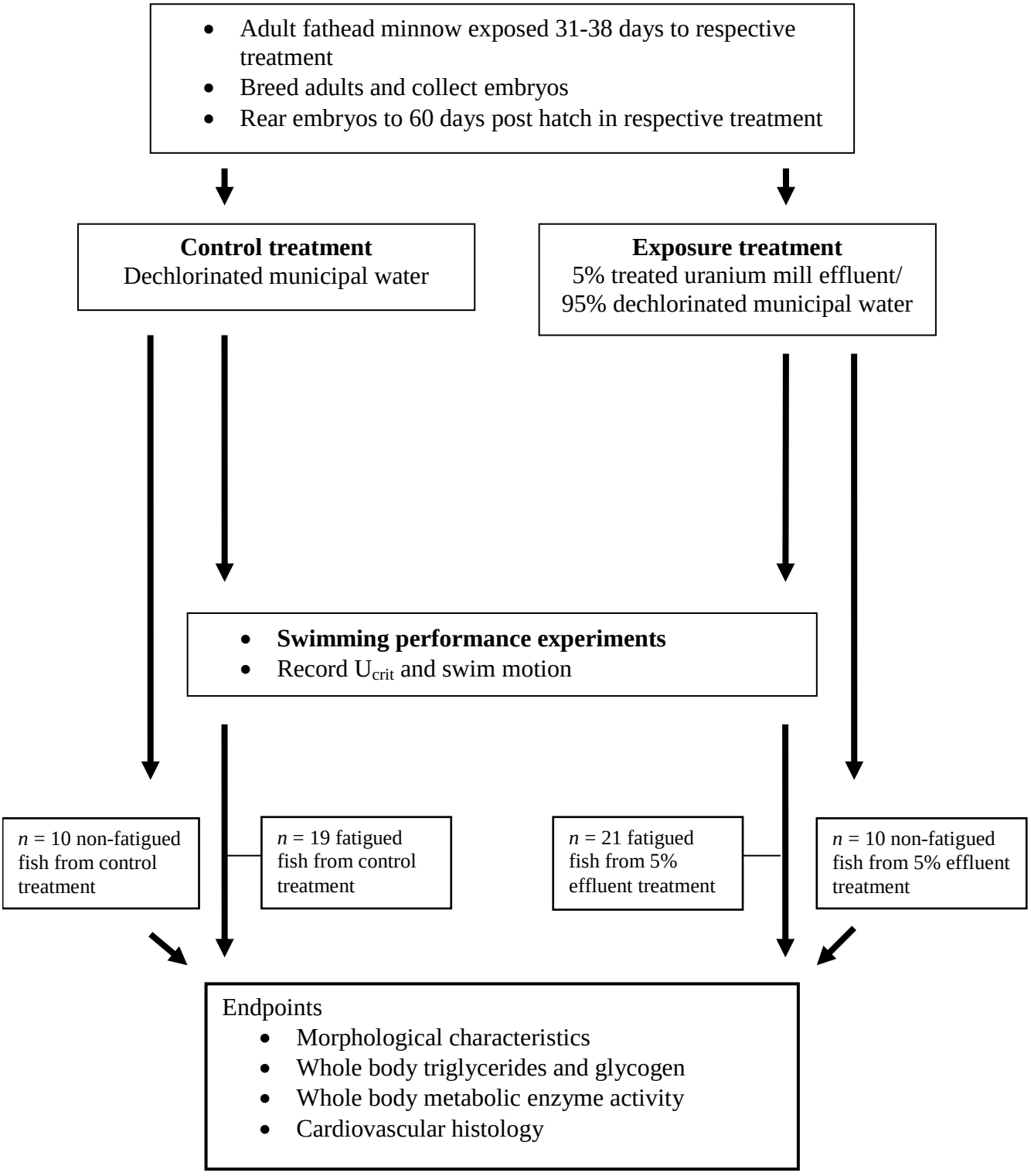

Figure 1.7 Flow chart of laboratory project experimental design for fall 2009, Saskatoon, Saskatchewan.

CHAPTER 2
2.0 METHOD DEVELOPMENT

Over the course of this thesis it was necessary to develop or modify two assay protocols in order to test tissue activity of citrate synthase (CS) and β–hydroxyacyl coenzyme A dehydrogenase (HOAD). Accurate determination of CS activity required the modification of a commercial kit. However, no commercial kit was available for the determination of tissue HOAD activity. Therefore, a HOAD assay protocol was adapted from previous studies and optimized for shiner muscle and FHM whole body samples.

2.1 Citrate synthase assay

2.1.1 Introduction

Citrate synthase is present in virtually all eukaryotic cell mitochondria and plays a central role in aerobic organism metabolism (Wiegand and Remington, 1986). This enzyme is observed primarily within the mitochondrial matrix where it catalyzes the first reaction of the Krebs cycle, which is a rate-limiting step. The key reaction is as follows:

$$\text{Acetyl-CoA} + \text{Oxaloacetate} + H_2O \rightarrow \text{Citrate} + \text{CoA} + H^+$$

Normally in the mitochondria citrate is then converted to *cis*-aconitate which continues through the Krebs cycle (for diagram refer to Figure 1.5). Because of its key role in aerobic metabolism, quantification of this enzyme activity in tissues involved in swimming can provide information on tissue aerobic scope, and overall organism aerobic capacity (Rajotte and Couture, 2002; Lemos et al., 2003).

The commercially available CS activity kit is based on the following reactions:

1) Acetyl-CoA + Oxaloacetate → Citrate + CoA + H^+

2) CoA + 5,5'-dithiobis-(2-nitrobenzoic acid) → 5-thio-2-nitrobenzoic acid + CoA-S-S-(5-thio-2-nitrobenzoic acid)

Reaction 2) involves the coenzyme A-thiol reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB). This latter product generates a yellow colour, the absorbance of which can be measured spectrophotometrically at 412 nm. This assay is marketed for use in purified cell lines, mitochondrial preparations and some mammalian tissue preparations (Sigma Aldrich, 2011). However, it may be unsuitable for use with tissue media that contain variable levels of endogenous thiols and/or coenzyme A (such as tissue or whole body organism homogenates) that could react non-specifically with DTNB (Else et al., 1988). Alternatively, some endogenous tissue enzymes could also have deacetylation capabilities, which would affect acetyl coenzyme A levels and could also contribute to non-specific activity. As a result, a citrate synthase-specific inhibitor is desirable to calculate specific CS activity, particularly when using this assay in crude tissue homogenates. Previous studies have shown palmitoyl coenzyme A is a reliable citrate synthase-specific inhibitor in a wide range of organisms, including single-celled organisms like *Escherichia coli* and *Bacillus megaterium* (Tubbs, 1963; Else et al., 1988) or porcine heart (Tubbs, 1963; Else et al., 1988).

Therefore, the objective of this study was to modify an existing CS assay for use in whole body fish and muscle homogenates. An integral element of this objective was to account for endogenous reactive components in homogenate preparations that could exhaust DTNB reserves or react with added acetyl coenzyme A. To accomplish this objective the utility of palmitoyl

coenzyme A as a specific CS inhibitor was investigated. Once determined, optimal homogenate concentrations for this assay were determined to ensure optimal assay performance.

2.1.2 Methods and materials

2.1.2.1 Basic assay conditions

Enzyme activities were determined in approximately 100 mg shiner muscle obtained immediately lateral to the dorsal fin or whole FHM (60 dph). Samples were thawed on ice, weighed, diluted 4× with homogenizing buffer (100 mM Tris, 3 mM EDTA, 2 mM $MgCl_2$, 1 mM reduced glutathione, 0.1% peroxide- and carbonyl-free Triton-X100; pH 7.8), minced with scissors and homogenized on ice (5×15s) using a PowerGen Model 125 homogenizer (Fisher Scientific, Ottawa, ON). Immediately prior to assaying, all homogenates were centrifuged at 1000×g at 4°C for 5 minutes to remove any insoluble tissues and supernatants were used in assays.

A CS activity kit was purchased (Sigma Aldrich, Oakville, ON; catalogue #CS0720). For all tests enzyme activity was determined in a 96 well plate with the following reagent volumes per well, as specified by the kit protocol with slight modification:

5 µL tissue homogenate or purified porcine heart CS (positive control)

181 µL assay buffer

2 µL 30 mM acetyl coenzyme A

2 µL 10 mM DTNB solution

22 µL 10 mM palmitoyl coenzyme A or water vehicle

10 µL 10 mM oxaloacetate

All reagents except tissue homogenate and palmitoyl coenzyme A were provided in the kit. Palmitoyl coenzyme A was made fresh daily from an aliquoted stock solution prepared

earlier, maintained at -80°C. All reagents were kept on ice until approximately 5 minutes prior to the assay in order to allow all solutions to warm to room temperature. Linear enzyme activity was measured at 412 nm at 22°C every 30 seconds over 1.5 minutes. The extinction coefficient of TNB at 412 nm was 13.6. All other assay details, including reagent preparation and activity calculations, were carried out as described in the kit product insert (Sigma Aldrich, 2010). For all tests activity was determined using 96-well plates in a SpectraMAX 190 spectrophotometer (Molecular Devices Corp, Sunnyvale, CA).

2.1.2.2 Determination of non-specific assay activity and optimal homogenate concentration

Initial tests determined non-specific assay activity in undiluted homogenate of pooled age-matched untreated FHM (*n*=1 sample, consisting of 3 pooled 60 dph municipal water fish) and purified porcine heart CS (Sigma Aldrich) as positive control (*n*=1 sample). Samples were assayed in the presence or absence of palmitoyl coenzyme A (100 mM) in triplicate. All other protocol details were as described above.

Subsequent tests determined optimal shiner muscle homogenate concentration for this assay. Briefly, muscle homogenate of shiner collected from the reference and shiner collected from the exposure lakes (see Chapter 3 of this thesis for details of site location and collection) and were assayed at 10× dilution and compared to undiluted samples (n=2 fish per lake). Muscle samples were run in triplicate in the presence or absence of palmitoyl coenzyme A (100 mM). Based on the prior test (investigating non-specific assay activity) that used undiluted homogenate of pooled age-matched FHM from control water, it was determined unnecessary to test diluted homogenates of individual FHM from 5% effluent and control treatments as undiluted pooled homogenate yielded suitable activity.

2.1.2.3 Calculations

Due to limited assay reagents and sample volumes, lower numbers of replicates were used during assay modification tests. As a result, data could not be analysed for statistical significance. Specific activity was determined for each sample by subtracting non-specific absorbance (measured in presence of palmitoyl coenzyme A) from total absorbance (measured in absence of pamitoyl coenzyme A).

2.1.3 Results

Pure CS enzyme (positive control) exhibited linear absorbance change at 412 nm over 1.5 minutes (Figure 2.1). However, the addition of 100 mM palmitoyl coenzyme A completely inhibited this enzyme activity. Whole body FHM positive control homogenate exhibited relatively linear absorbance change that was slightly lower than purified enzyme (Figure 2.1). The addition of 100 mM palmitoyl coenzyme A reduced absorbance change to approximately one third of uninhibited FHM homogenate activity.

Absorbance change was similar in muscle homogenate from shiner collected from both sites (Figures 2.2A, B). In each case shiner muscle homogenate diluted 10× had very low absorbance change over 1.5 minutes compared to undiluted stocks (Figures 2.2A, B). The addition 100 mM palmitoyl coenzyme A inhibited activity in shiner muscle from both sites in undiluted and 10× diluted homogenates.

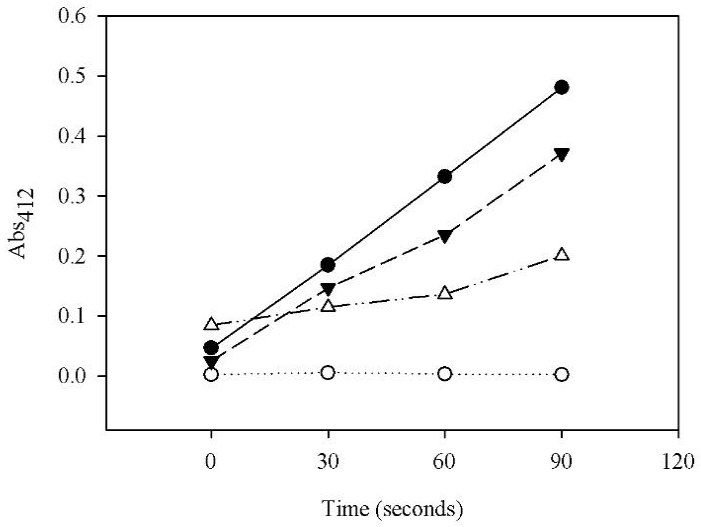

Figure 2.1 Purified citrate synthase (*n*=1 replicate) and undiluted homogenate of pooled age-matched FHM (*Pimephales promelas*; *n*=1 sample, consisting of 3 pooled 60 dph fish raised in dechlorinated municipal water) absorbance at 412 nm in the presence or absence of 100 mM palmitoyl coenzyme A over 90 seconds. Activity of purified citrate synthase in the absence (●) and presence of 100 mM palmitoyl coenzyme A (○) are shown. Also, pooled FHM whole body homogenate in the absence (▼) and presence of 100 mM palmitoyl coenzyme A (△) are shown. Samples were tested under each condition in triplicate.

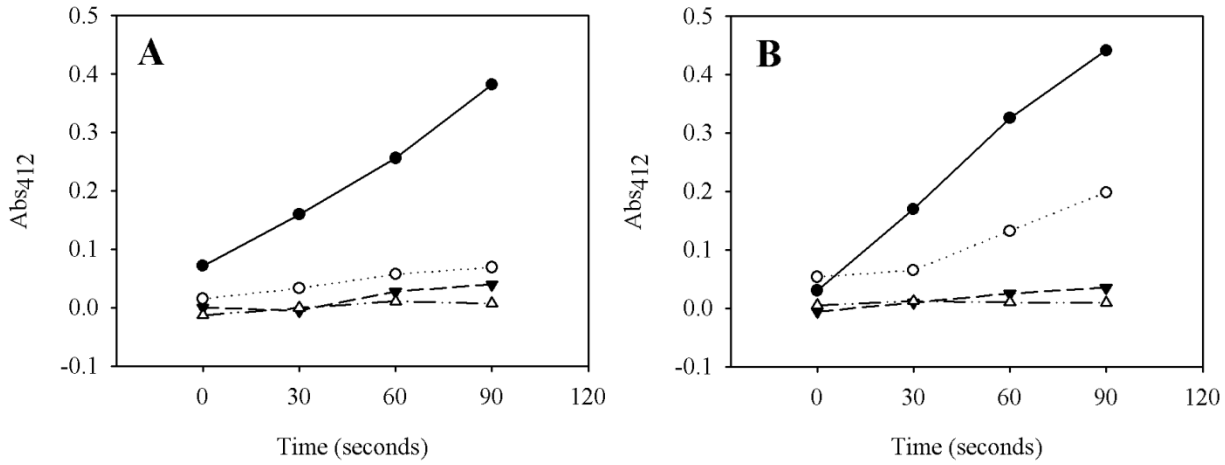

Figure 2.2 Optimization of sample concentration for spottail shiner (*Notropis hudsonius*) muscle homogenate in citrate synthase assay. Representative activities over time of undiluted (●, ○) and 10× diluted (▼, △) shiner muscle homogenate at 412 nm in the presence (○,△) and absence of 100 mM palmitoyl coenzyme A (●,▼) are shown. A: Muscle homogenate from a shiner collected from the reference lake (*n*=1 fish). B: Muscle homogenate from a shiner collected from the exposure lake (*n*=1 fish). Sample absorbance shown are mean values of triplicates determined for each assay condition or sample.

2.1.4 Discussion

Overall, 100 mM palmitoyl coenzyme A appeared to inhibit specific CS activity with non-specific activity evident in tissue and whole body homogenates. Optimal shiner muscle and whole body FHM homogenate activity was observed when samples were assayed undiluted which suggests homogenization protocols are suitable for obtaining tissue homogenate of adequate concentration. However future studies that require more concentrated homogenate may need to modify the existing homogenization protocol. Overall, this assay is useful for the assessment of CS activity in fish tissue homogenates provided a CS specific inhibitor is used in parallel samples.

Although 100 mM palmitoyl coenzyme A completely inhibited purified porcine CS, the possibility that CS activity was not completely inhibited in whole body FHM or shiner muscle homogenates cannot be excluded. However, previous studies have demonstrated that palmitoyl coenzyme A inhibits enzyme activity in a wide range of organisms such as bacteria and certain mammals with similar affinity (Tubbs, 1963; Srere, 1968; Else et al., 1988). Thus, the concentration of palmitoyl coenzyme A used in this thesis should also be effective in completely inhibiting fish CS activity. Therefore, it was assumed in the final protocol for this assay that CS activity in samples containing 100 mM palmitoyl coenzyme A was fully inhibited. This assumption could be more precisely confirmed in future experiments if shiner or FHM CS was purified, then tested in the presence and absence of palmitoyl coenzyme A.

The commercial assay kit that was purchased is based on deacetylation of acetyl coenzyme A and was designed for testing activity in whole cell extract and isolated mitochondria. The results obtained in this study demonstrate non-specific activity in tissue homogenates partly contributed to sample absorbance change during tests. It is plausible other

enzymes in the homogenates also have deacetylation capabilities, which could have been responsible for non-specific activity observed in samples containing 100 mM palmitoyl coenzyme A. Overall, this study illustrates the importance of investigating the limitations of commercial assays if one is using samples other than those for which the kit has been validated. Future studies using this assay to test CS activity in tissue homogenate should employ an enzyme-specific inhibitor discussed here as this CS assay (without the inclusion of inhibitors) may provide misleading results of CS activity in tissue homogenates. Further research investigating non-specific activity in tissue homogenates alongside cell extracts and isolated mitochondria is warranted.

2.2 β-hydroxyacyl coenzyme A dehydrogenase assay

2.2.1 Introduction

β-hydroxyacyl coenzyme A dehydrogenase (HOAD) is a rate-limiting enzyme involved in mitochondrial β–oxidation of fatty acids. Fatty acid β–oxidation is the cyclic enzyme-catalyzed process by which fatty acids are catabolised by removing two carbons units (acetyl coenzyme A) per cycle (for diagram refer to Figure 1.4). Although β–oxidation systems are present in both mitochondria and peroxisomes, the bulk of metabolic activity (for tissue ATP production) originates from mitochondrial processes (Moyes and West, 1995; Yang and He, 1999). β-hydroxyacyl coenzyme A dehydrogenase catalyzes the rate-limiting second dehydration step in fatty acid β–oxidation.

β-hydroxyacyl coenzyme A dehydrogenase is present in various tissues, and its activity is especially elevated in aerobic muscles with metabolic preference for fatty acids, such as cardiac and red muscle (Yang and He, 1999). This enzyme is highly evolutionarily conserved, with activity observed in organisms ranging from *E. coli* to humans as well as numerous fish species

(Binstock and Schultz, 1981; Fernandez et al., 1999; Leonard, 1999; Yang and He, 1999; Rajotte and Couture, 2002). Recent work in humans has highlighted different mitochondrial HOAD enzymes that vary with fatty acid chain length specificity, including short-chain, long-chain, and medium-chain specific enzymes (reviewed by Yang et al., 2005). The majority of mitochondrial activity, at least in humans (Yang et al., 2005) and *E. coli* (Binstock and Schultz, 1981) is due to medium-chain length fatty acid activity. Fatty acid β–oxidation in fish has received far less attention than in mammalian systems and still remains unclear on many levels (Moyes and West, 1995), but there is little evidence to suggest β–oxidation in fish varies substantially from mammals.

In mitochondria, the physiological reaction catalyzed by HOAD (in both fish and mammals) is as follows:

$$\beta\text{-hydroxyacyl-CoA} + NAD^+ \rightarrow \beta\text{-ketoacyl-CoA} + NADH$$

However, most protocols for measuring HOAD activity are based on the methods of Bradshaw and Noyes (1975), which uses the reaction:

$$\text{Acetoacetyl-CoA} + NADH \rightarrow \beta\text{-hydroxyacyl-CoA} + NAD^+$$

In this method HOAD activity is measured by monitoring the decrease in NADH, but runs in the opposite direction to the normal physiological process (He et al., 1989). While it may be counterintuitive to measure the activity of an enzyme in the reverse direction, this protocol is nonetheless useful for quantifying maximal enzyme activity in comparative studies, including the

present study using fish tissues (Fernandez et al., 1999; Leonard, 1999; Londraville and Duvall, 2002; Rajotte and Couture, 2002). Clearly, for studies in which *in vivo* activity rates are desired, quantifying enzyme activity in the physiological direction is essential (He et al., 1989).

The reverse-direction HOAD assay indirectly measures HOAD activity by measuring NADH disappearance spectrophotometrically at 340 nm. Most assays measuring HOAD activity in fish tissue using this protocol are thought to assess optimal β-oxidation of short to medium-chain length fatty acids (Pelletier et al., 1994; Fernandez et al., 1999; Leonard, 1999; Rajotte and Couture, 2002). There are no commercially available kits to measure HOAD activity, although basic assay methodology for the quantification of HOAD activity (in the physiologically reverse direction) has been reported for several fish species (Pelletier et al., 1994; Londraville and Duvall, 2002; Rajotte and Couture, 2002). Therefore, a HOAD assay protocol developed from these previous studies required a series of optimization steps in order to ensure accurate assessment of maximal enzyme activity in shiner muscle and FHM whole body homogenate. Furthermore, NADH is photosensitive and can degrade with time once in solution. As a result it was imperative that methods for this assay prevented or delayed NADH breakdown during assaying. Lastly, no known inhibitors of HOAD exist (BRENDA, 2011). Since both acetoacetyl CoA and NADH can be non-specifically consumed in various pathways in tissue homogenates (*e.g*. NADH oxidation in the electron transport chain, acetoacetyl CoA integration in the mevalonate pathway; Goldstein and Brown, 1990) it was important to develop a method that would correct for non-specific activity. Therefore, the approach taken to assess specific HOAD activity was to compare sample activity values to those in the presence and absence of the assay substrate (acetoacetyl coenzyme A).

The main objective of this study was to develop an HOAD assay that determined specific activity in both spottail shiner muscle and whole body FHM homogenate. It was desirable to have a final assay that was sensitive to relatively low activity levels in small amounts of tissue, but that was also cost- and time-effective. To attain these results, optimal assay conditions were determined over a series of steps:

1) Investigate NADH stability and photosensitivity at different concentrations over time to ensure NADH concentrations remain sufficiently elevated during assaying.

2) Determine optimal assay acetoacetyl coenzyme A (substrate) concentration in shiner muscle and FHM whole body homogenates.

3) Determine optimal assay homogenate dilution in shiner muscle and FHM whole body homogenates.

4) Determine optimal assay NADH concentration in shiner muscle and FHM whole body homogenates.

2.2.2 Materials and Methods

2.2.2.1 Basic assay conditions

Enzyme activities were determined in the same shiner muscle and FHM whole body homogenate stocks used for CS assay optimization. All samples were kept at -80°C until immediately prior to assaying, at which point homogenates were thawed on ice and centrifuged for 5 minutes at 1000 rpm for 4°C to remove any tissue fragments and connective tissue. For all tests, activity was determined using 96-well plates in a SpectraMAX 190 spectrophotometer (Molecular Devices Corp, Sunnyvale, CA).

All wells contained 100 mM Tris/1 mM EDTA assay buffer (pH 7.4). Also, all wells contained 1 mM KCN (final well concentrations), which was added to inhibit mitochondria

NADH-depleting enzymes other than HOAD. Specifically, cyanide inhibits cytochrome oxidase (Complex IV) in the mitochondrial electron transport chain, consequently inhibiting electron transport and non-specific NADH oxidation. Optimal homogenate, NADH, and acetoacetyl coenzyme A concentrations were determined in separate tests (discussed below). A stock of 100 mM Tris/1 mM EDTA assay buffer (pH 7.4) was made ahead of time and kept at 4°C. NADH and KCN stocks were made fresh daily using assay buffer and kept on ice, protected from light.

All assay reagents for each individual tissue homogenate sample that was tested, except the substrate acetoacetyl coenzyme A, were pre-incubated at 22°C in the absence of light in microcentrifuge reaction tubes for 10 minutes. This preincubation step was determined to be necessary in preliminary experiments to ensure completed inhibition of oxidative processes by KCN that could interfere with assay activity. Microcentrifuge tubes were gently inverted to mix contents until homogeneous before and after incubation. Samples were assayed in triplicate. The assay reaction was then initiated with the addition of acetoacetyl coenzyme A (the concentration of which varied with each reagent optimization step) with an equivalent volume of water as the vehicle control. Activity was measured every minute for 10 minutes at 340 nm at 25°C. 96-well plates were shaken between measurements. The extinction coefficient of NADH was 6.22.

2.2.2.2 NADH stability under assay conditions

Prior to the optimization of assay reagents, NADH stability under assay conditions was determined. NADH absorbance at various concentrations was measured at 340 nm in the presence of all assay reagents except sample homogenates. Crystalline NADH powder was reconstituted with assay buffer and added to 96-well plate immediately prior to absorbance measurements in order to minimize time between reconstitution and measurements. Final well concentrations typical of a standard linear curve were tested: 0.013, 0.025, 0.050, 0.100, 0.200,

0.400 mM NADH. Two standard curves were plated in triplicate, each on opposite sides of a 96-well plate. Absorbance was measured once every minute for 10 minutes, once at 30 minutes, and once at 90 minutes. Between measurements, the plate was removed from the spectrophotometer and one standard curve was covered with aluminum foil, and the other was covered with a clear plate cover at room temperature.

2.2.2.3 Optimal substrate concentration determination

Optimal substrate (acetoacetyl coenzyme A) concentration was determined in shiner muscle homogenate and whole body FHM homogenate. As a starting point, FHM whole body and shiner muscle samples were first diluted 5× with assay buffer. The NADH concentration was maintained at 0.16 mM. In contrast, acetoacetyl coenzyme A concentrations tested were 0.025 mM, 0.05 mM, and 0.10 mM (final well concentrations) based on the range used in previous fish white muscle studies report (Pelletier et al., 1994; Rajotte and Couture, 2002) in triplicate. All other protocol steps were carried out as described above (Section 2.2.2.1).

2.2.2.4 Optimal homogenate concentration determination

Once optimal acetoacetyl coenzyme A concentrations were determined, optimal sample homogenate concentrations were determined. Shiner muscle homogenate and whole body FHM homogenate were diluted 10×, 5×, 3× with ice cold assay buffer or left undiluted. Samples were assayed in triplicate as described above in both the presence and absence of substrate (Section 2.2.2.1).

2.2.2.5 Optimal NADH concentrations determination

Once optimal homogenate dilutions were determined, the optimal NADH concentration was determined. NADH concentrations tested (0.12, 0.16, 0.20 mM NADH, final well concentrations) were obtained from the range used in previous HOAD studies in fish tissue

(Pelletier et al., 1994; Rajotte and Couture, 2002). During assays a single NADH stock solution was made with ice cold assay buffer immediately prior to assay. This method was selected over weighing out NADH for three separate stocks in order to minimize the amount of NADH waste during stock preparation and to avoid weight measurement errors. For these tests, shiner muscle homogenate was diluted 5× and FHM whole body homogenate was diluted 3×. All other protocol steps were carried out as described above and sample activity was determined in the presence or absence of substrate (Section 2.2.2.1).

2.2.2.6 Calculations

Due to limited assay reagents and sample volumes, low numbers of replicates were used during assay testing. As a result, data could not be analysed for statistical significance. Specific activity was determined by subtracting the linear absorbance change in vehicle-control wells (non-specific activity) from wells containing acetoacetyl coenzyme A (total activity).

2.2.3 Results

2.2.3.1 NADH stability under assay conditions

NADH absorbance did not appear to substantially decrease with time or light exposure (Figure 2.3A, B). At almost all concentrations in both light and dark conditions there was a small decrease in absorbance within the first minute, but between 2 and 10 minutes absorbance only changed minimally or remained constant (Figure 2.3A, B). After 30 minutes, absorbance appeared to increase slightly in wells containing higher NADH concentrations (0.100, 0.200, 0.400 mM NADH) that were both covered with foil or plate covers. However, after 90 minutes absorbance decreased in all concentrations to equal or slightly below 30 minute levels.

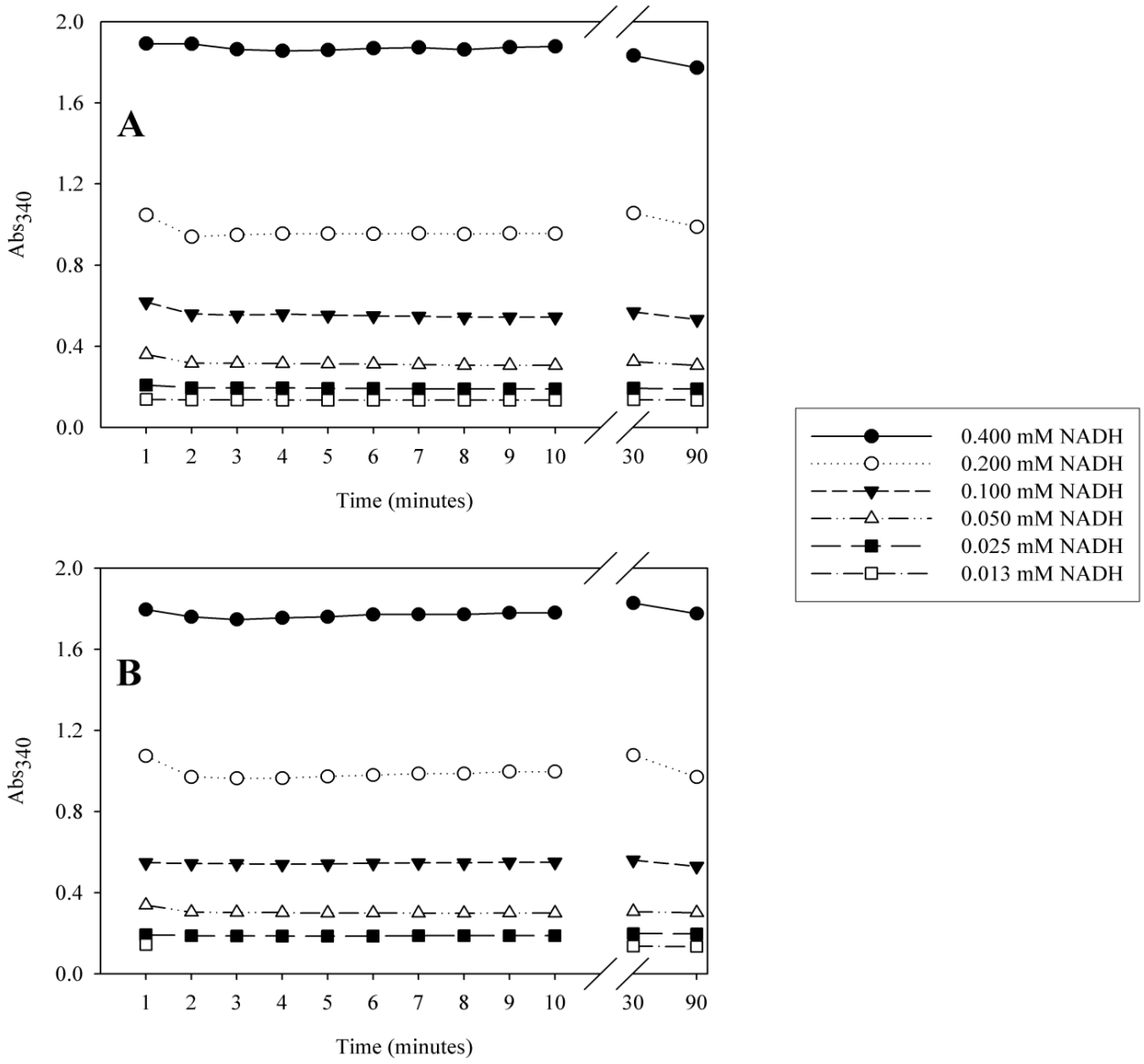

Figure 2.3 NADH standard curve concentration absorbance at 340 nm over 90 minutes in the absence (A) or presence (B) of light. Values are from standards assayed in duplicate in the presence of all HOAD assay reagents except sample homogenates. Absorbance was measured once every minute for 10 minutes, once at 30 minutes, and once at 90 minutes after NADH was added to wells.

2.2.3.2 Acetoacetyl coenzyme A concentration optimization

Absorbance decreased in both shiner muscle (*n*=1-2 fish per lake; Figure 2.4A-F) and whole body FHM (*n*=1-2 fish per treatment; Figure 2.5A-F) homogenates at all acetoacetyl coenzyme A concentrations over 10 minutes. In whole body FHM and shiner muscle, linear segments of activity were greatest with 0.100 mM acetoacetyl coenzyme A, intermediate at 0.050 mM acetoacetyl coenzyme A, and lowest at 0.025 mM acetoacetyl coenzyme A. Activity between shiner muscle from the reference and exposure lakes appeared to be similar at all substrate concentrations. Assay activity appeared to be lower in FHM from the 5% effluent treatment compared to control. Vehicle-control (*i.e*. without substrate) well absorbance for some shiner muscle and FHM whole body homogenate samples decreased slightly over 10 minutes. This observation could be the result endogenous oxidative pathways that were not inhibited by KCN, or other processes that involve NADH. This could also explain some of the variability observed other in samples (both with and without acetoacetyl coenzyme A). Overall, optimal assay activity occurred in both shiner muscle and whole body FHM homogenate in the presence of 0.100 mM acetoacetyl coenzyme A.

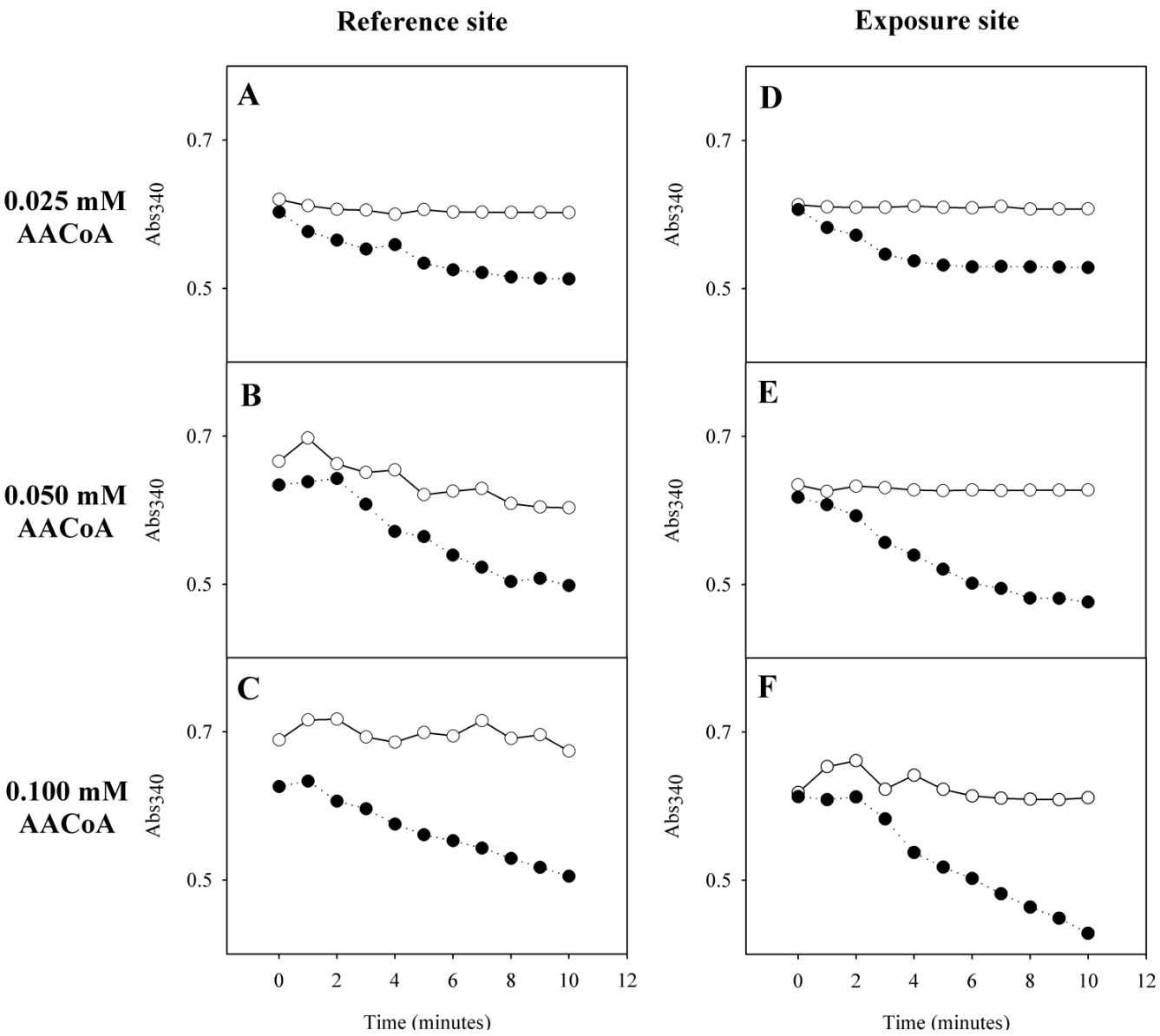

Figure 2.4 Representative figures of the effect of acetoacetyl coenzyme A concentration on 5× diluted spottail shiner (*Notropis hudsonius*) muscle homogenate absorbance at 340 nm. Values are the mean of triplicates determined in a single shiner muscle homogenate from the reference lake (A, B, C) or from the exposure lake (D, E, F). Samples were tested in the presence (●) or absence (○) of 0.025 mM (A, D), 0.050 mM (B, E), or 0.100 mM (C, F) acetoacetyl coenzyme A over 10 minutes at 340 nm.

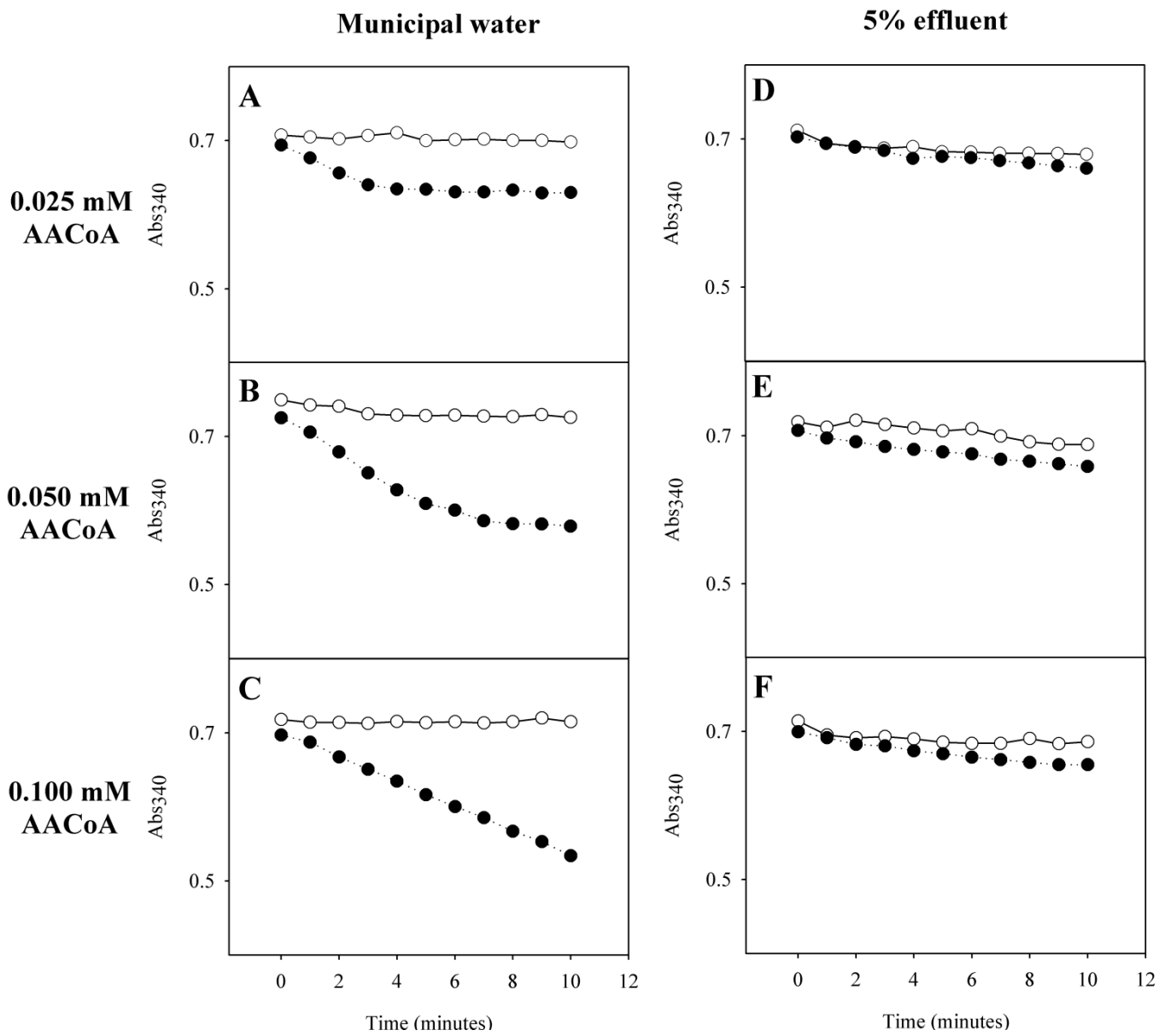

Figure 2.5 Representative figures of the effect of acetoacetyl coenzyme A concentration on 5× diluted whole body fathead minnow (*Pimephales promelas*) homogenate absorbance at 340 nm. Values are the mean of triplicates determined in a single fathead minnow whole body homogenate reared in the control water treatment (A, B, C) or the 5% effluent treatment (D, E, F). Samples were tested in the presence (●) or absence (○) of 0.025 mM (A, D), 0.050 mM (B, E), or 0.100 mM (C, F) acetoacetyl coenzyme A over 10 minutes at 340nm.

2.2.3.3 Homogenate concentration optimization

Based on the range used in previous studies, preliminary studies in this thesis investigated HOAD activity in homogenates diluted 100× and 50× (Pelletier et al., 1994; Levesque et al., 2002; Rajotte and Couture, 2002; Couture, P., personal communication). However, absorbance changes at these dilute homogenate concentrations were negligible. Therefore, subsequent optimization experiments in this thesis tested homogenate dilutions of 10×, 5×, 3× and undiluted samples.

In shiner muscle homogenate (*n*=1-2 fish per lake), linear absorbance change over 10 minutes increased with increasing homogenate concentration (Figure 2.6A, B). Results were comparable between shiner collected from the reference lake (Figure 2.6A) and from the exposure lake (Figure 2.6B). As an example, only HOAD activity for samples diluted 3× are shown in Figures 2.6A and 2.6B, but HOAD activities for all samples diluted 5× and 10× followed similar trends (data not shown). Overall, 3× diluted samples yielded more consistent results compared to 5× or 10× diluted samples. Based on these results HOAD activity was further examined in undiluted versus 3× diluted shiner muscle (*n*=1-2 fish per lake). However, undiluted shiner muscle homogenate from fish from both lakes produced inconsistent absorbance curves and a lower percent of specific HOAD activity (Figures 2.7A, B). Therefore, 3× dilution of shiner muscle homogenate was chosen for use in subsequent assays.

FHM whole body homogenate activity (*n*=1-2 fish per treatment) over 10 minutes also increased with increasing homogenate concentration in both fish from the control municipal water treatment (Figure 2.8A) and from the 5% effluent treatment (Figure 2.8B). Again, although non-specific activity (open triangles; samples without acetoacetyl coenzyme A added) is only shown for samples diluted 3× in Figures 2.8A and 2.8B for reference, non-specific activities for

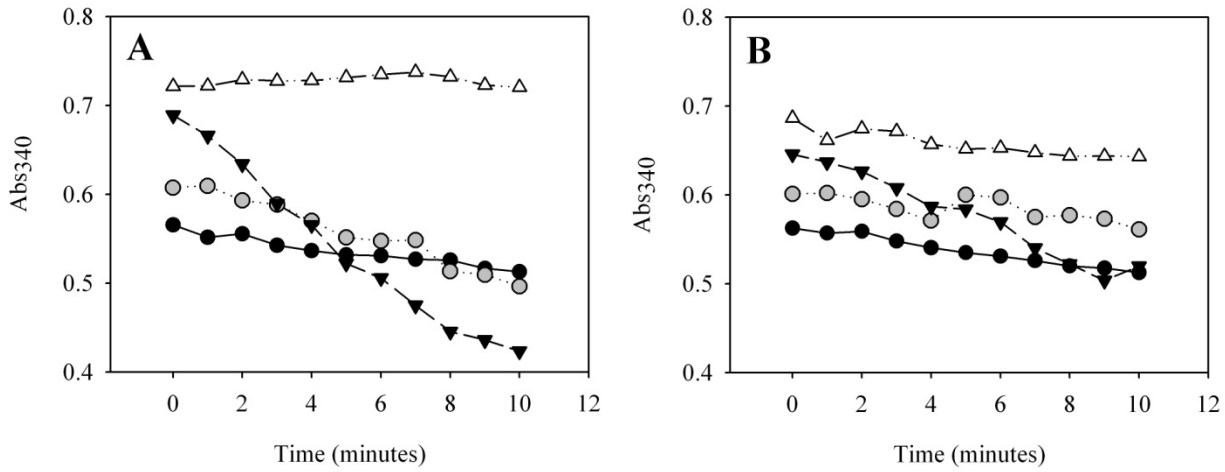

Figure 2.6 Representative figures of the effect of muscle homogenate concentration on the absorbance at 340 nm in spottail shiner (*Notropis hudsonius*) collected from the reference lake (A; *n*=1-2 fish) or the exposure lake (B; *n*=1-2 fish) in the β-hydroxyacyl coenzyme A dehydrogenase assay. Muscle homogenate was diluted 10× (●), 5× (●), or 3× (▼) in the presence of 0.100 mM acetoacetyl coenzyme A. 3× diluted muscle sample was also assayed in the absence of acetoacetyl coenzyme A (△). All sample conditions were plated in duplicate.

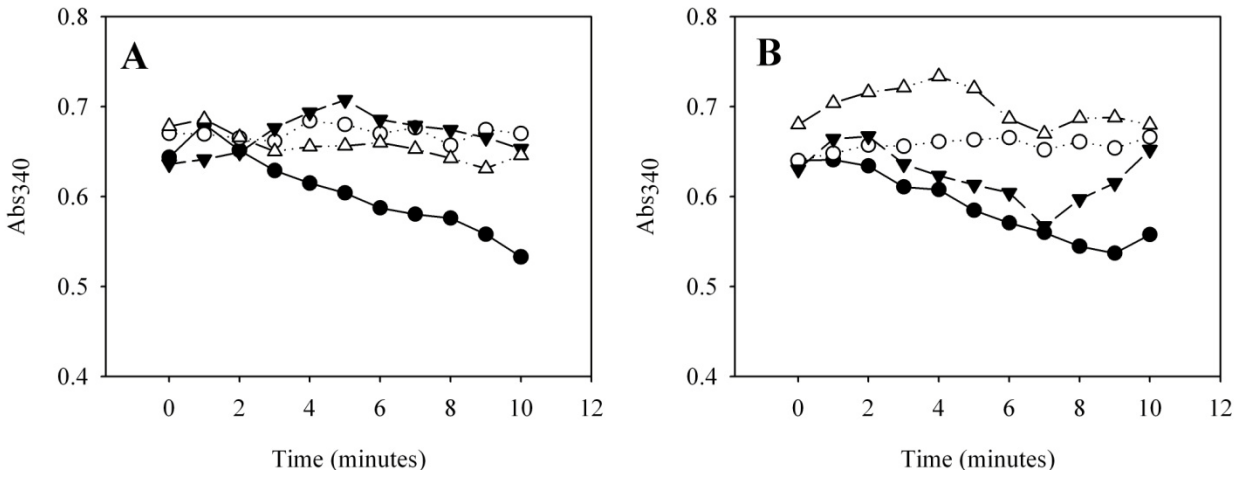

Figure 2.7 Representative figures comparing activity in 3× diluted (circles) or undiluted (triangles) muscle homogenate from spottail shiner (*Notropis hudsonius*) collected from the reference lake (A; *n*=1 fish) or the exposure lake (B; *n*=1 fish) in the β-hydroxyacyl coenzyme A dehydrogenase assay. Samples were assayed in the presence (closed symbols) or absence (open symbols) of 0.100 mM acetoacetyl coenzyme A over 10 minutes at 340 nm. All samples were plated in duplicate.

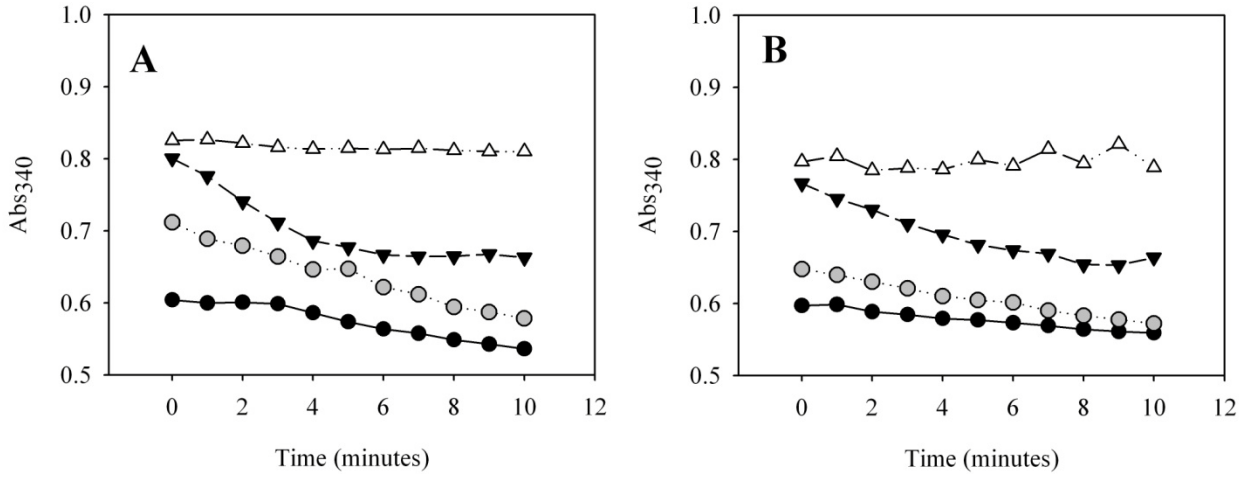

Figure 2.8 Representative figures of the effect of whole body homogenate concentration on the absorbance at 340 nm in fathead minnow (*Pimephales promelas*) from the control water treatment (A; *n*=1 fish) or the 5% effluent treatment (B; *n*=1 fish) in the β-hydroxyacyl coenzyme A dehydrogenase assay. Whole body homogenate was diluted 10× (●), 5× (●), or 3× (▼) in the presence of 0.100 mM acetoacetyl coenzyme A. 3× diluted whole body homogenate was also assayed in the absence of acetoacetyl coenzyme A (△). All samples were plated in duplicate.

all samples diluted 5× and 10× followed similar trends (data not shown). Diluting FHM homogenate 3× rather than 5× or 10× ensured maximal linear reaction rate of the assay within the 10 minutes.

2.2.3.4 NADH concentration optimization

Since NADH is both the coenzyme and analyte in the HOAD assay, NADH concentration affected baseline absorbance in both shiner muscle homogenate (Figures 2.9A-F) and FHM whole body homogenate (Figures 2.10A-F). In shiner muscle homogenate, initial absorbance was approximately 0.5 at 0.12 mM NADH concentration (Figures 2.9A, D) at 340nm, approximately 0.6 at 0.16 mM NADH (Figures 2.9B, E), and slightly below 0.8 at 0.20 mM NADH (Figures 2.9C, F). In FHM whole body homogenate, initial absorbance was approximately 0.7 in 0.12 mM NADH (Fig. 2.10A, D), approximately 0.85 at 0.16 mM NADH (Figures 2.10B, E), and slightly below 1.0 at 0.20 mM NADH (Figures 2.10C, F). There was no clear effect of NADH concentration on enzyme activity in shiner muscle or FHM whole body homogenate. However, at the highest NADH concentration (0.20 mM NADH), samples appeared to have greater variability than at lower NADH concentrations, especially in FHM homogenate. However, it is unclear why variability would increase at higher NADH concentrations (Figures 2.9C, F; 2.10C, F), but may be due to the relatively high absorbance (~1.0 at 340 nm). Since there was relatively little difference in activity between the lowest (0.12 mM NADH) and intermediate (0.16 mM NADH) concentrations, 0.16mM NADH was selected for subsequent HOAD assay conditions to better ensure NADH did not become limiting.

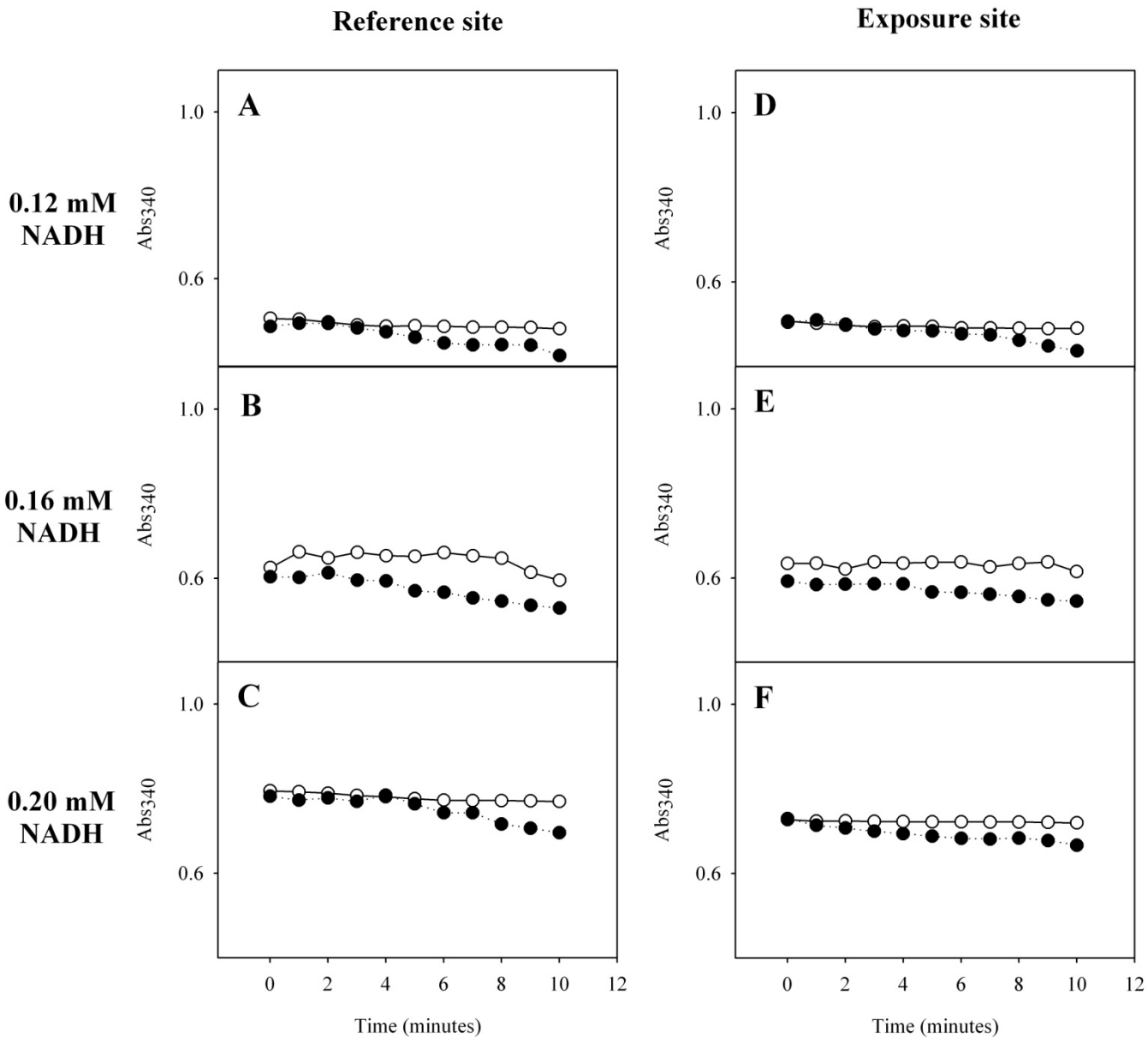

Figure 2.9 Representative figures of the effect of NADH concentration on 5× diluted spottail shiner (*Notropis hudsonius*) muscle homogenate absorbance at 340 nm over 10 minutes. Values are from single replicates of muscle homogenate from one shiner from the reference lake (A, B, C) or one shiner from the exposure lake (D, E, F) assayed in triplicate. Samples were tested using 0.12 mM (A, D), 0.16 mM (B, E) or 0.20 mM (C, F) NADH. Traces represent activity in wells in the presence (closed circles ●) or absence (open circles ○) of 0.100 mM acetoacetyl coenzyme A.

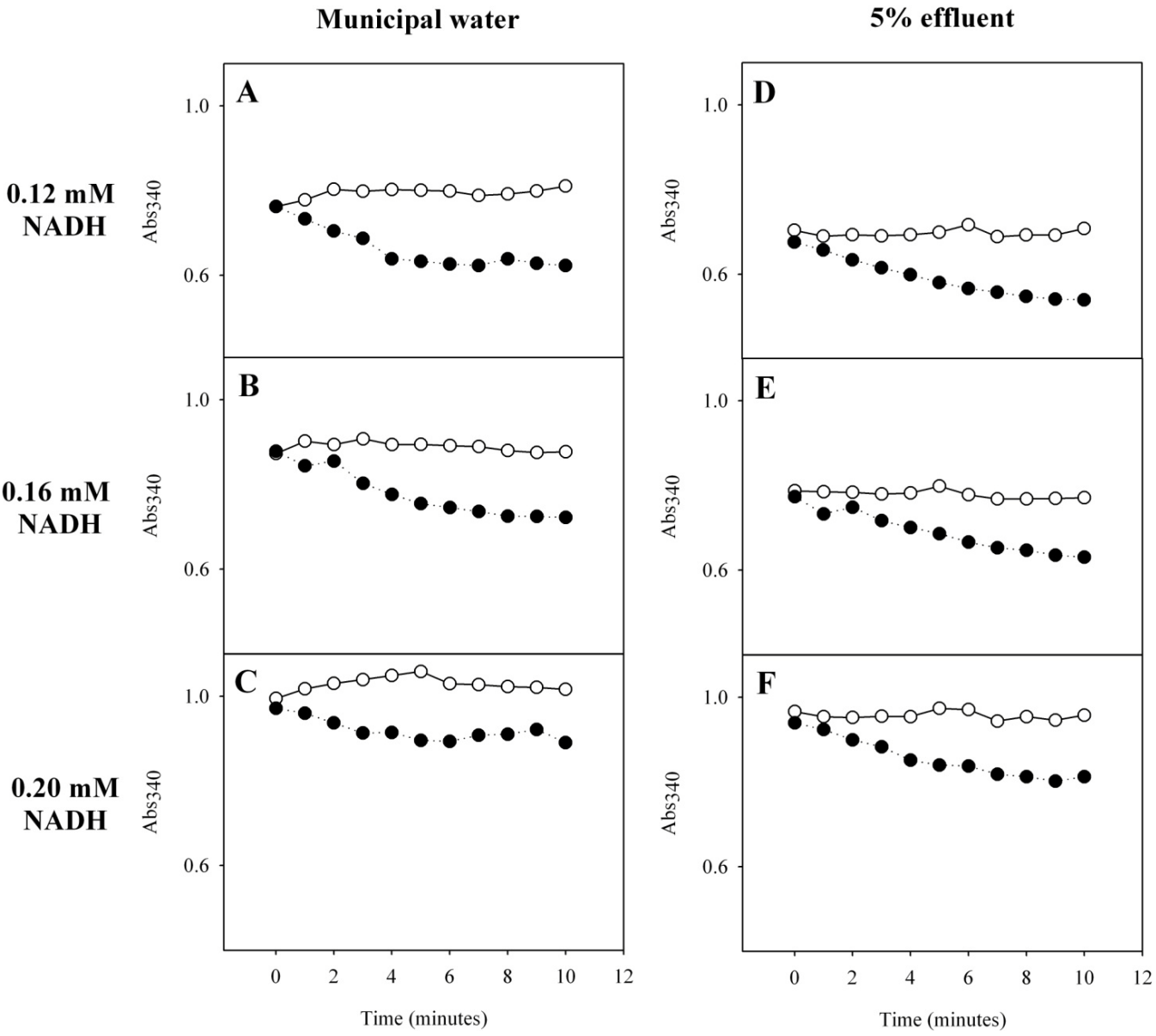

Figure 2.10 Representative figures of the effect of NADH concentration on 3× diluted whole body fathead minnow (*Pimephales promelas*) homogenate absorbance at 340 nm over 10 minutes. Values are from single replicates of whole body homogenate from one fathead minnow from the control water treatment (A, B, C) or one fathead minnow from the 5% effluent treatment (D, E, F) assayed in triplicate. Samples were tested using 0.12 mM (A, D), 0.16 mM (B, E) or 0.20 mM (C, F) NADH. Traces represent activity in the presence (●) or absence (○) of 0.100 mM acetoacetyl coenzyme A.

2.2.4 Discussion

The purpose of this study was to develop an assay that assessed maximal HOAD activity suitable for use in both shiner muscle and whole body FHM homogenates. In developing this assay, three reagent concentrations were optimized (acetoacetyl coenzyme A, homogenate, and NADH concentrations). The final assay conditions utilized 0.16 mM NADH in the presence or absence of 0.100 mM acetoacetyl coenzyme A. Furthermore, it was shown whole body FHM homogenate or shiner muscle homogenate, both diluted 3× yielded optimal assay activity.

In the present study, NADH did not appear to substantially degrade non-enzymatically within 30 minutes. Therefore, the person performing experiments has reasonable time between NADH reconstitution and reaction initiation. This is especially true if reagents remained on ice and in the dark. Furthermore, NADH is supplied in sufficient excess to negate effects of any minor drop in NADH concentration.

Overall, assay reagent concentrations and activity rates in this thesis closely resemble those reported in previous studies in different fish species (Pelletier et al., 1994; Londraville and Duvall, 2002; Rajotte and Couture, 2002). In the present study, assay parameters were similar for both shiner white muscle and whole body FHM. Since white muscle generally accounts for approximately 85-95% body mass, white muscle would account for the majority of whole body homogenate content (Moyes and West, 1995). Furthermore, the similarity in optimal assay conditions between shiner muscle and FHM tissues in this thesis and in previous studies suggests similar assay conditions could be applied to other species or tissues with different metabolic capacities (Guderley and Gawlicka, 1992). As a result potential may exist for a common HOAD assay for various fish tissues (such as the commercial CS kit), but further validation of the assay protocols in a wider variety of species or tissues is needed.

Discrepancies exist on the recommended temperature for this assay. Some studies suggest optimal assay activity occurs at room temperature (20-25°C; Fernandez et al., 1999; Leonard, 1999; Londraville and Duvall, 2002; Rajotte and Couture, 2002), while others suggest most relevant data is generated when this assay is performed at fish physiological temperatures (10-15°C; Guderley and Gawlicka, 1992; Pelletier et al., 1994). Unpublished data suggests maximal HOAD activity in fish muscle occurs at or slightly below room temperature (Couture, P., personal comunication). The purpose of most HOAD protocols in the literature discussed above is to characterize maximal HOAD activity, but physiologically-relevant temperatures will produce the most environmentally-meaningful data. This thesis used room temperature for the HOAD assay, but future studies could use the same temperature in which the fish were housed to better assess *in vivo* rates of fatty acid β-oxidation.

The incorporation of an HOAD-specific inhibitor in future studies could also be useful to better characterize specific HOAD activity in crude samples. Currently there are reports of specific inhibitors for short-chain and long-chain HOAD enzymes in humans (Yang et al., 2005), but there is no known specific inhibitor for medium-chain HOAD in humans or other species (BRENDA, 2011). Alternatively, a further improvement to this assay would be the inclusion of a HOAD positive control. However, it appears most commercially available stocks of active HOAD are purified from human tissue and/or very expensive. Thus, current positive control sources are inappropriate for an assay examining fish HOAD activity. Future studies should investigate isolation and purification techniques of active HOAD from fish tissue based on the protocols described for swine heart (Bradshaw and Noyes, 1975) and *E. coli* (Binstock and Schultz, 1981).

CHAPTER 3
3.0 SWIMMING PERFORMANCE AND ENERGY HOMEOSTASIS IN SPOTTAIL SHINER (*NOTROPIS HUDSONIUS*) COLLECTED DOWNSTREAM OF A URANIUM MILL

3.1 Introduction

Integrated physiological traits in fish, such as swimming performance and metabolic status can provide more biologically insightful information associated with contaminant exposure than traditional environmental and morphometric endpoints (Rajotte and Couture, 2002; McKenzie et al., 2007). Fish survival in the wild strongly depends on swimming ability as it relates to activities such as feeding, predator evasion, migration and mating (Beaumont et al., 1995; Drucker, 1996; Plaut, 2001). To characterize swimming performance a number of protocols based on Brett's Critical swimming speed (U_{crit}; Brett, 1964) have been developed. This endpoint has provided an ecologically relevant assessment of swim endurance in many fish species (reviewed by Plaut, 2001). In addition to swim endurance, swim motion characteristics, such as tail beat frequency and tail beat amplitude, can be used to quantify the manner in which a fish swims and its relation to metabolism (Steinhausen et al., 2005; Tudorache et al., 2010).

Maximal oxygen consumption occurs at or immediately prior to U_{crit}, suggesting cardiorespiratory performance also plays a key role in determining swimming performance (Farrell, 2007). Some fish species maximize oxygen carrying capacity by increasing hematocrit during activity or in hypoxic environments (Butler et al., 1992; Gallaugher et al., 1995; Robb and Abrahams, 2003; Sandblom and Axelsson, 2007). Similarly, cardiac output and cardiac morphology have been shown to strongly influence swimming ability (Claireaux et al., 2005; Farrell, 2007).

During slow or routine swimming, and for swimming speeds up to approximately 80% U_{crit}, aerobic metabolism predominates in fish muscle (Webb, 1971a). Triglycerides account for

the bulk of oxidizable substrates fuelling aerobic swimming, although protein can serve as an alternate fuel source once lipid pools are exhausted (reviewed by Moyes and West, 1995). At speeds greater that 80% U_{crit}, or burst swimming most energy is derived from anaerobically metabolized intramuscular glycogen. As a result, lactate is generated in muscle during burst swimming, which enters systemic circulation and serves as a measurable endpoint of anaerobic metabolic activity.

Tissue aerobic and anaerobic capacity can be characterized by assessing the activity of rate-limiting enzymes involved in metabolic pathways. Citrate synthase (CS) is a key enzyme in the citric acid cycle, and is considered an indicator of tissue aerobic ability (Rajotte and Couture, 2002; Lemos et al., 2003). Alternatively, β–hydroxyacyl coenzyme A dehydrogenase (HOAD) is involved in β–oxidation of lipids, the activity of which can be indicative of tissue lipolytic ability (Londraville and Duvall, 2002; Rajotte and Couture, 2002). While these enzymes are primarily involved in aerobic metabolism some anaerobic activity could play a role in routine swimming (Moyes et al., 1992; Rajotte and Couture, 2002). The acute physiological stress response is also closely related to metabolic responses during swimming. Catecholamines and cortisol are released as a part of this response and cause metabolic and cardiorespiratory changes (Jobling, 1994). This leads to increased hematocrit, plasma lactate, and mobilized glycogen stores, which may coincide with exercise-related changes in metabolism.

Critical swimming speed is a sensitive endpoint that has been shown to be affected by many environmental contaminants, including ammonia (reviewed by McKenzie et al., 2003), dissolved metals (Wilson and Wood, 1992; Beaumont et al., 1995; Alsop et al., 1999; McGeer et al., 2000; Rajotte and Couture, 2002; Taylor et al., 2004) and complex mixtures such as coal ash (Hopkins et al., 2003), crude oil fractions (Kennedy and Farrell, 2006) or urban river systems

(McKenzie et al., 2007). Some environmental contaminants have the ability to cause subtle developmental effects in juvenile or adult life stages that could significantly reduce swimming and/or cardiovascular ability. For example, selenium is known to cause cardiovascular and morphological terata in maternally exposed fish larvae (Holm et al., 2005; Muscatello et al., 2006). Morphological deformities such as spinal curvatures could also impair swim motion characteristics (tail beat frequency or tail beat amplitude) while cardiovascular abnormalities could impair oxygen delivery to aerobic tissues.

Chronic trace element exposure is reported to alter the acute stress response and energy homeostasis in some northern fish species. For example, wild yellow perch (*Perca flavescens*) from metal-contaminated lakes have reduced muscle enzyme activities (CS, HOAD, lactate dehydrogenase; Rajotte and Couture, 2002), altered seasonal energetic stores (Levesque et al., 2002) and impaired cortisol production (Brodeur et al., 1997; Laflamme et al., 2000; Levesque et al., 2002). Some of these effects have been reported in fish downstream of the Key Lake uranium mill (northern Saskatchewan, Canada), which discharges complex effluent into the local water system. Previous studies reported that certain fish species downstream of the mill have higher triglyceride and higher glycogen stores than fish from reference lakes (Bennett and Janz, 2007; Kelly and Janz, 2008). No difference was observed in prey triglyceride levels, but fish collected from the exposure site had significantly lower parasite abundance, which offers a possible partial explanation for the observed increased energy stores (Kelly and Janz, 2008). Characterizing the swimming performance and energy homeostasis in fish downstream of the Key Lake uranium mill could thus provide insight into previously described bioenergetic effects.

The objective of this study was to investigate the potential effects of chronic exposure to components of metal mining wastes on swimming performance and energy homeostasis in a wild

minnow species, spottail shiner (*Notropis hudsonius*). To evaluate these effects, shiner were collected from a lake downstream of the Key Lake uranium mill, or from a nearby ecologically similar reference lake. Fish from each lake were either swam to fatigue in on-site incremental velocity (U_{crit}) tests, or withheld from U_{crit} tests (non-fatigued). In addition to swimming performance, trace element body burdens, basic biometrics (fork length, mass, condition factor, hepatosomatic index), biochemical measures of energy stores (liver and muscle glycogen and triglycerides), metabolic pathway endpoints (hematocrit, plasma glucose and lactate, and muscle metabolic enzyme activity), and cardiovascular structures (bulbus arteriosus, ventricle, dorsal aorta diameter) were compared between fish collected from each lake.

3.2 Materials and Methods

3.2.1 Study site, trace element analysis, and fish collection

The Key Lake uranium mill is located in northern Saskatchewan, Canada (57°13'N, 105°38'W) and is situated on the David Creek drainage system (for map see Muscatello et al., 2006). In 2006, this mill discharged approximately 135,130 m^3 treated effluent/month (which is known to contain variable, but generally higher concentrations of arsenic, molybdenum, nickel, selenium, uranium, thallium, ammonia and organics) into the local drainage system (Golder Associates, 2008). The exposure site (Delta Lake) is located approximately 11 km downstream of the effluent discharge point. The reference site (Yeoung Lake) is located approximately 7 km south-east of the mill site and lies within a separate drainage system.

General water chemistry (pH, conductivity, total dissolved solids, salinity, and temperature) were assessed on-site using a multi-parameter YSI probe (6 series - YSI Inc., Yellow Springs, OH). Temperature was measured on three separate days during fish captures. All other basic water chemistry parameters in the present study were based on a single sample

taken during fish collection. Juvenile spottail shiner were collected from both lakes mid-June 2009. Shiner were obtained using beach seines, which were used to isolate and capture schools of shiner in 3-4 different locations of both lakes. Capture success was similar between lakes. Captured shiner were kept in holding nets in resident lakes for up to 48 hours. On the day of swimming tests, shiner were transported approximately 10 km from resident lakes to the experiment site in coolers containing fresh aerated lake water. Water temperature was maintained below 15°C. All fish were treated in accordance with protocols approved by the University of Saskatchewan Animal Research Ethics Board and the Canadian Council on Animal Care guidelines on experimental animal care and use.

3.2.2 Swim performance tests

Immediately prior to swimming tests shiner were removed from holding coolers and weighed. Critical swimming speed was evaluated in individual shiner using a LoligoSystems Mini Swim Tunnel (LoligoSystems, Tjele, Denmark) with circulating fresh oxygenated water from the corresponding lake. Circulating water temperature was maintained at 12°C, which reflected the average ambient lake temperature at the time of collection. The swim tunnel apparatus was filled with approximately 50 L of circulating lake water. Shiner were acclimated in the swim chamber for 45 minutes at low flow (approximately 1 body length/s). During swimming tests, water velocity was increased stepwise in 5 minute increments by 5 cm/s (*i.e.* velocity increased approximately 1 body length/increment) until fatigue. This protocol was directly adapted from the protocols of Dussault et al. (2008) and Kaufmann (1990), which were modified to accommodate early life-stage and/or small bodied fish in small swim tunnels. Critical swimming speed was calculated as: $U_{crit} = V_p+((t_f/t_i)\times V_i)$, where: V_i is the velocity increase per increment; V_p is the final velocity swam; t_i is the increment time length; t_f is the

duration of the last velocity increment until fatigue. Because fish occupied greater than 5% volume of the swim tunnel, a solid blocking coefficient was calculated for each fish and applied to final U_{crit} values (Bell and Terhune, 1970).

Swim motion was recorded concurrently with a high speed camera (250 frames/s) to enable measurement of tail beat frequency and tail amplitude using Midas 2.0 imaging software. Both endpoints were calculated at the highest completed velocity interval during U_{crit} testing. Tail beat frequency was analyzed using Adobe Premiere Elements 2.0, while video frames for tail amplitude analysis were selected using this software and then exported for analysis with Image-Pro 6.0. Tail amplitude was calculated as the maximum deviation distance of the most posterior point of the caudal peduncle from the longitudinal body line (for both right and left body strokes), which was averaged over three separate tail beat cycles for each shiner.

For each capture a second group of shiner from the same cohort were withheld from swimming tests, but subjected to the same penning and transportation as shiner that underwent swim trials. These shiner were euthanized without swimming in U_{crit} tests (non-fatigued) and metabolic endpoints were compared to fatigued shiner.

3.2.3 Biometrics and sample collection

Fish were euthanized using an overdose of Aquacalm (approximately 5 mg metomidate hydrochloride/L; Syndel Laboratories Ltd., Qualicum Beach, BC) immediately after swimming performance experiments. Fish were visually inspected for gross deformities characteristic of selenium exposure, including skeletal curvatures, craniofacial deformities, and fin deformities. Fork-length was recorded, then blood for hematocrit and plasma analysis was collected in heparinised capillary tubes via caudal severance and centrifuged for 15 minutes in a capillary centrifuge to obtain plasma. Muscle samples were excised from the muscular area of the fish

between the lateral line and dorsal fin, slightly posterior to the dorsal fin. Muscle, liver and plasma samples were immediately frozen on dry ice and stored at -80°C upon return to the University of Saskatchewan. Condition factor was calculated as: $CF = [(100 \times \text{weight})/(\text{length}^3)]$. Hepatosomatic index (HSI) was calculated as: HSI = [(100×liver weight)/(body weight-liver weight)]. Hematocrit was calculated by dividing the red blood cell column length in the capillary tube by the total blood column length after centrifugation. Age was determined based on length-frequency of year class data from a previous study (Golder Associates, 2008).

Heart and caudal peduncle samples, and gonads if visible, were preserved in Bouin's fixative for 24-48 hours and stored in 70% ethanol until sectioning and staining. Tissue was sectioned serially with 5 μm cross sections, then eosin/hematoxylin stained. All cardiovascular endpoints were assessed at 50× magnification, and gonads at 200× magnification, using an Axio Observer.Z1 inverted microscope (Carl Zeiss MicroImaging, LLC, Goettingen, Germany) and measured using digital image analysis software (AxioVision LE). Bulbus arteriosus muscle diameter and ventricle diameter were measured in 3 consecutive sections (with 50 μm tissue between each section) at the maximum chamber width and averaged for each fish. Dorsal aorta inner diameter was measured at the point within the caudal peduncle where the vertebrate-to-body length ratio equalled 1.58 ± 0.05%, and averaged over three consecutive sections. Shiner sex was determined in gonads based on the presence or absence of visible ovarian follicles (oocytes). If oocytes were visible, fish were confirmed female and maturity was assessed based on the presence or absence of previtellogenic, vitellogenic, and mature follicles. Gonads from shiner in which oocytes were absent were designated males.

3.2.4 Trace element analysis

For whole body trace element analysis, individual non-fatigued shiner (*n*=5 shiner per lake) were euthanized using an overdose of Aquacalm and were immediately frozen on dry ice and stored at -80°C upon return to the University of Saskatchewan. Whole fish were freeze dried for 72-96 hours and ground using a porcelain mortar and pestle for subsequent tissue digestion. Sample moisture content was calculated as: % moisture = [100×(wet mass-dry mass)/(wet mass)]. For each fish, approximately 0.05 g dried samples were cold digested in Teflon vessels using 5 mL of ultra-pure nitric acid and 1.5 mL of hydrogen peroxide (30%, Suprapur, EMD Chemicals, Gibbstown, NJ). Digested samples were evaporated in Teflon vessels at approximately 65˚C and then reconstituted in 5 mL of 2% nitric acid. Prior to analysis reconstituted samples were syringe filtered using 0.45 µm polyethersulfone filters to remove any particulate matter. Trace element concentrations in whole body fish (µg/g dry weight) were determined using inductively coupled plasma-mass spectrometry (ICP-MS) at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK) with a certified reference material (TORT-2, Lobster hepatopancreas) obtained from the National Research Council of Canada (Ottawa, ON) as described previously (Phibbs et al., 2011).

3.2.5 Laboratory analysis

3.2.5.1 Tissue energy stores and plasma assays

All reagents were purchased from Sigma Aldrich (Oakville, ON) unless otherwise specified. Triglyceride content was determined in liver and dorsal muscle samples as previously described (Weber et al., 2003). Liver and muscle glycogen content were determined using amylase with a glucose oxidase-based kit with slight modifications (Weber et al., 2008). Briefly, samples were first incubated with amylase at 60°C for 2 hours, then digested samples were

assayed for free glucose. Plasma glucose was determined using a similar protocol without enzyme incubation. Lactate was determined using a kit purchased from Eton Bioscience (Eton Bioscience Inc., San Diego, CA).

3.2.5.2 Enzyme activity

Enzyme activities were determined in approximately 100 mg of muscle. Briefly, muscle samples were thawed on ice, weighed, diluted 4-fold with homogenizing buffer (100 mM Tris, 3 mM EDTA, 2 mM $MgCl_2$, 1 mM reduced glutathione, 0.1% peroxide- and carbonyl-free Triton-X100; pH 7.8), minced with scissors and homogenized on ice (5×15s) using a PowerGen Model 125 homogenizer (Fisher Scientific, Ottawa, ON). Immediately prior to assaying, all homogenates were centrifuged at 1000×g at 4°C for 5 minutes to remove any insoluble tissues.

Citrate synthase activity was determined in muscle homogenates (*n*=8 non-fatigued fish, *n*=14-16 fatigued fish per site) using a commercially available kit (Sigma Aldrich, Oakville, ON). Specific activity was determined by assaying each sample in the presence or absence of palmitoyl coenzyme A (100 mM), a CS specific inhibitor, in triplicate. Enzyme activity was measured at 412 nm at 22°C over 1.5 minutes. Specific activity was determined for each sample by subtracting non-specific absorbance from total absorbance. Citrate synthase enzyme activity was expressed as International Units (IU) per gram of tissue (μmoles citrate liberated per minute per gram tissue).

Preliminary experiments determined optimal β–hydroxyacyl coenzyme A dehydrogenase (HOAD) activity conditions in muscle homogenate (*n*=4-5 non-fatigued fish, *n*=11-13 fatigued fish per site) based on protocols of Rajotte and Couture (2002) and Pelletier et al. (1994). Activity was determined by measuring the oxidation of NADH at 340 nm at 25°C for 10 minutes under the following conditions: 100 mM Tris, 1 mM EDTA, 1 mM KCN, 0.16 mM NADH; pH

7.4. Samples were assayed in triplicate in the presence or absence of acetoacetyl coenzyme A (0.1 mM). Specific activity was determined by subtracting the linear absorbance change in vehicle-control wells (endogenous activity) from wells containing acetoacetyl coenzyme A. β–hydroxyacyl coenzyme A dehydrogenase enzyme activity was expressed as IU per gram of tissue (μmoles NADH oxidized per minute per gram tissue).

3.2.6 Statistical analysis

Data were tested with Shapiro-Wilk normality test and Bartlett's test for homogeneity of variance. Data that failed these tests were log10 transformed to achieve normality and homogeneity of variance. Some trace element body burdens and plasma lactate failed parametric assumptions despite transformation, and were tested non-parametrically using Mann-Whitney U tests or Kruskal-Wallis tests, respectively. Significant differences in fork length, wet weight, trace element body burdens, U_{crit} (body length/s), absolute U_{crit} (cm/s), tail beat frequency and amplitude, condition factor, hepatosomatic index, and cardiovascular endpoints (standardized to body weight) were detected using a *t*-test. Two-way analysis of variance (ANOVA) was used to analyze swim and metabolism data with site (reference vs. exposure) and swim status (non-fatigued vs. fatigued) as factors, followed by Least Square Difference posteriori tests. There were no interactions between factors for all two-way ANOVAs. Results were considered significant if $p<0.05$. Data are reported as mean ± standard error of the mean (SEM).

3.3 Results

3.3.1 Water chemistry and trace element body burdens

Temperature and pH were comparable between lakes, while conductivity, total dissolved solids, and salinity were elevated in the exposure lake (Table 3.1). Reference and exposure site ammonia, hardness, sulphate and carbonate concentrations in a previous study are included in

Table 3.1 Water chemistry parameters of reference (Yeoung Lake) and exposure (Delta Lake) sites mid June 2009 at Key Lake milling operation, Saskatchewan.

Parameter	Reference site	Exposure site
Temperature (°C)	12.2 ± 2.2	12.3 ± 4.3
Conductivity (µS/cm)	12	405
pH	6.95	6.56
Total dissolved solids (mg/L)	0.01	0.31
Salinity (mg/L)	0.01	0.23
Ammonia (mg/L)*	0.1	0.6
Hardness (mg/L)*	6	254
Sulphate (mg/L)*	0.9	250.0
Carbonate (mg/L)*	<1	<1

All values except ammonia were measured mid-June 2009.
All values, except temperature (as mean ± standard error of the mean; *n*=3 samples), were obtained from single water samples.
*Ammonia, hardness, sulphate and carbonate values are from Golder Associates (2008).

Table 3.1 for further comparison between lakes (Golder Associates, 2008). All parameters except carbonate concentration were consistently higher in the exposure lake. The moisture content was 83.6 ± 1.6% in fish collected from the exposure lake (n=5 fish), and 83.0 ± 0.8% for fish collected from the reference lake (n=5 fish; data not shown).

Whole body concentrations of boron (p<0.01), molybdenum (p<0.01), selenium (p<0.01), and thallium (p<0.01) were significantly greater in shiner collected from the exposure lake compared to shiner collected from the reference lake (Table 3.2). Barium (p<0.001), manganese (p<0.001), mercury (p<0.01), and strontium (p<0.001) were significantly lower in shiner collected from the exposure lake (Table 3.2). All other trace element body burdens were similar between lakes.

3.3.2 Fish morphometrics, cardiovascular structure and gonad histology

Fish collected from the exposure lake had significantly lower mass (1.20 ± 0.02 g; p<0.01) and fork length (4.9 ± 0.1 cm; p<0.01) compared to fish collected from the reference lake (mass 1.45 ± 0.07 g; fork length 5.2 ± 0.1 cm; Table 3.3). However, condition factor did not significantly differ between lakes. Hepatosomatic index was lower in shiner collected from the exposure lake (1.24 ± 0.06; p<0.01) compared to the reference lake (1.55 ± 0.07; Table 3.3). Despite size differences, all fish from both sites were verified young of previous year based on length-frequency data (Golder Associates, 2008). No gross deformities were observed in either group (Table 3.3).

Ventricle diameter (standardized to body weight) was significantly greater in shiner collected from the exposure lake (905.2 ± 17.4 μm/g; p<0.05) compared to the reference lake (783.6 ± 55.4 μm/g; Table 3.4 and Figure 3.1). There were no significant differences in body-weight standardized bulbus arteriosus muscle diameter or dorsal aorta inner diameter between

Table 3.2 Whole body trace element concentrations (µg/g dry weight; *n*=5 fish per site) in juvenile spottail shiner (*Notropis hudsonius*) collected mid June 2009 from reference (Yeoung Lake) and exposure (Delta Lake) sites at Key Lake uranium mill, Saskatchewan.

Analyte	Reference site	Exposure site	*p*
Aluminum	29.22 ± 6.33	40.52 ± 5.90	0.228
Arsenic	0.45 ± 0.07	0.38 ± 0.04	0.366
Barium	27.71 ± 2.66	1.85 ± 0.02	<0.001
Boron†	40.02 ± 1.98	230.24 ± 145.86	0.008
Cadmium	0.63 ± 0.18	0.71 ± .029	0.823
Chromium†	2.54 ± 1.39	1.08 ± 0.17	0.421
Cobalt	0.37 ± 0.04	0.41 ± 0.11	0.911
Copper	2.82 ± 0.12	2.62 ± 0.05	0.177
Iron	68.65 ± 6.49	58.37 ± 2.58	0.179
Krypton	3.39 ± 0.37	4.93 ± 0.58	0.055
Manganese	24.40 ± 3.02	7.59 ± 0.67	<0.001
Mercury	0.13 ± 0.01	0.06 ± 0.01	0.002
Molybdenum†	0.45 ± 0.01	1.39 ± 0.32	0.008
Nickel	0.88 ± 0.26	0.58 ± 0.09	0.292
Selenium†	2.24 ± 0.03	17.50 ± 1.25	0.008
Strontium	148.81 ± 5.33	17.92 ± 0.88	<0.001
Thallium†	0.09 ± 0.01	0.22 ± 0.07	0.008
Tin†	2.08 ± 1.74	0.21 ± 0.08	0.548
Uranium	0.03 ± 0.02	0.04 ± 0.01	0.916
Vanadium	0.05 ± 0.01	0.04 ± 0.01	0.194
Zinc	181.07 ± 26.34	139.69 ± 14.64	0.207

Data expressed as mean ± standard error of the mean. 9 degrees of freedom for all elements. Data was considered significant if $p<0.05$ using *t*-test or Mann-Whitney U test. *p* indicates *p*-values.
†Tested with Mann-Whitney U test.

Table 3.3 Morphometric data for juvenile spottail shiner (*Notropis hudsonius*) collected mid June 2009 from the reference (Yeoung Lake) and exposure (Delta Lake) sites at the Key Lake uranium milling operation, Saskatchewan.

Parameter	Reference site	Exposure site	*p*	d.f.	*F*
n	23	25			
Wet weight (g)	1.45 ± 0.07	1.20 ± 0.02	0.002	47	13.671
Fork length (cm)	5.2 ± 0.1	4.9 ± 0.1	0.004	47	9.303
Condition factor[a]	1.04 ± 0.03	1.02 ± 0.02	0.382	47	0.778
Hepatosomatic index[b]	1.55 ± 0.07	1.24 ± 0.06	0.002	44	10.967
Deformities	0	0			

Data expressed as mean ± standard error of the mean. Data was considered significant if $p<0.05$ using *t*-test. *p*: *p*-value; d.f.: degrees of freedom; *F*: *F*-statistic.

[a]Condition factor = [(100×weight)/(length^3)]. [b]Hepatosomatic index = [(100×liver weight)/(body weight-liver weight)].

Table 3.4 Ventricle diameter (*n*=6-7 fish per site) and bulbus arteriosus muscle diameter (*n*=5-7 fish per site; µm/g body mass), and dorsal aorta inner diameter (*n*=4 fish per site; µm/g body mass) of juvenile spottail shiner (*Notropis hudsonius*) collected mid June 2009 from reference (Yeoung Lake) and exposure (Delta Lake) sites at Key Lake uranium mill, Saskatchewan.

	Reference site	Exposure site	*p*	d.f.	*F*
Ventricle	783.6 ± 55.4	905.2 ± 17.4	0.046	12	5.044
Bulbus arteriosus	492.2 ± 44.8	509.9 ± 21.9	0.705	11	0.152
Dorsal aorta	16.3 ± 2.0	17.3 ± 1.4	0.702	7	0.161

Data expressed as mean ± standard error of the mean. Data was considered significant if $p<0.05$ using *t*-test. *p*: *p*-value; d.f.: degrees of freedom; *F*: *F*-statistic.

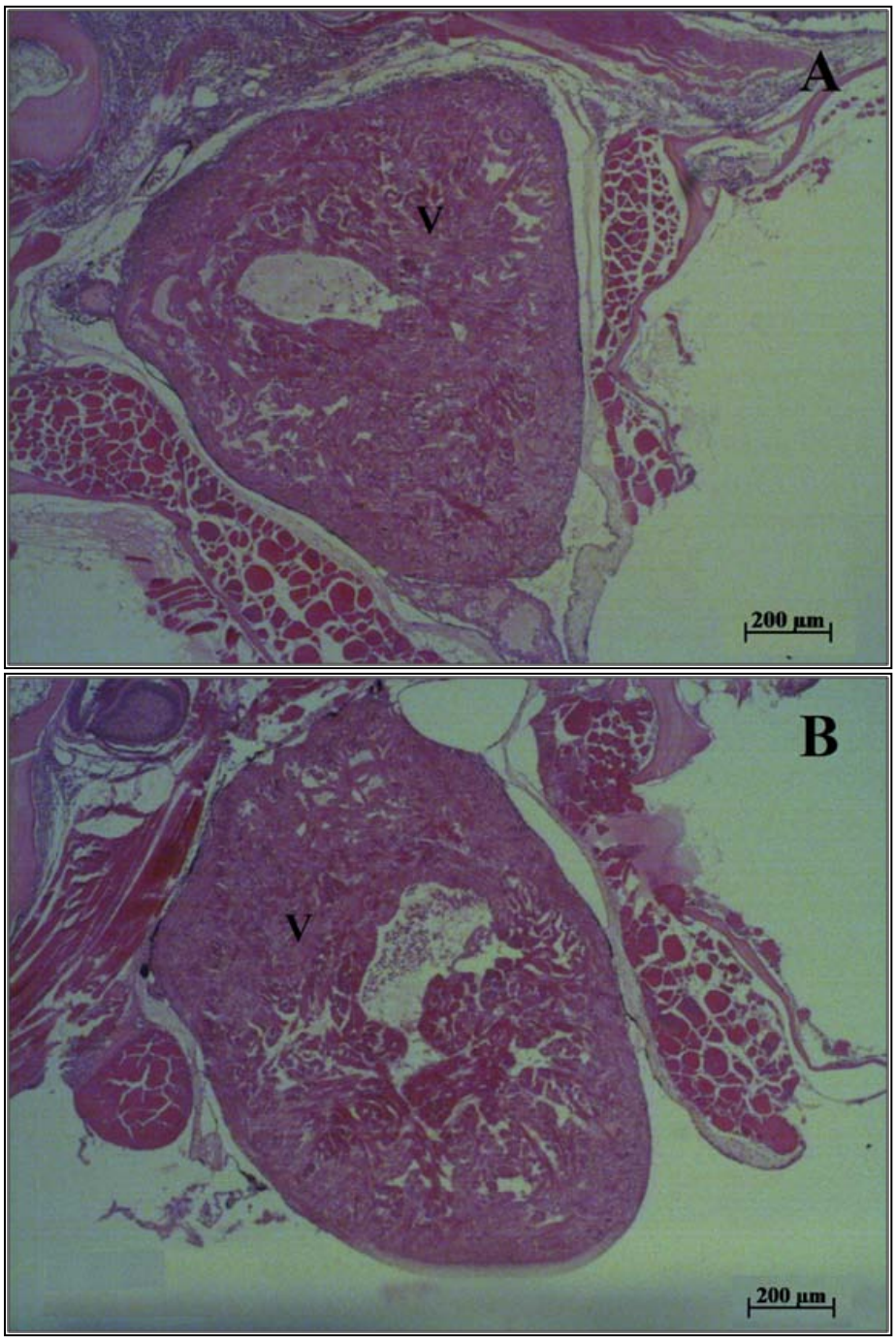

Figure 3.1 Representative images of hematoxylin/eosin stained sections of juvenile spottail shiner (*Notropis hudsonius*) ventricle from reference (A; Yeoung Lake) and exposure (B; Delta Lake) sites in June 2009 at Key Lake uranium mill, Saskatchewan. V, ventricle. Images are shown at approximately 50× magnification.

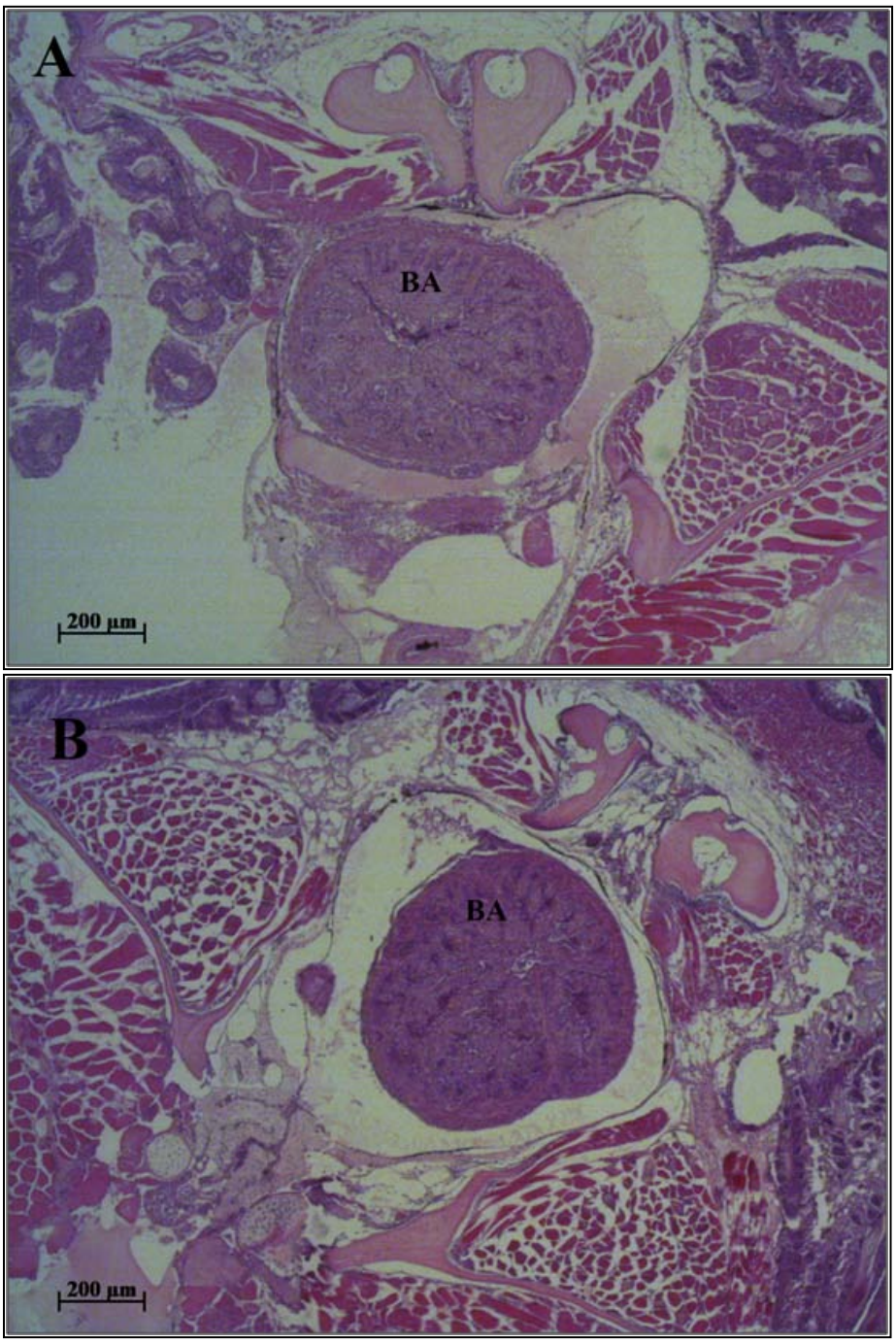

Figure 3.2 Representative images of hematoxylin/eosin stained sections of juvenile spottail shiner (*Notropis hudsonius*) bulbus arteriosus from reference (A; Yeoung Lake) and exposure (B; Delta Lake) sites in June 2009 at Key Lake uranium mill, Saskatchewan. BA, bulbus arteriosus. Images are shown at approximately 50× magnification.

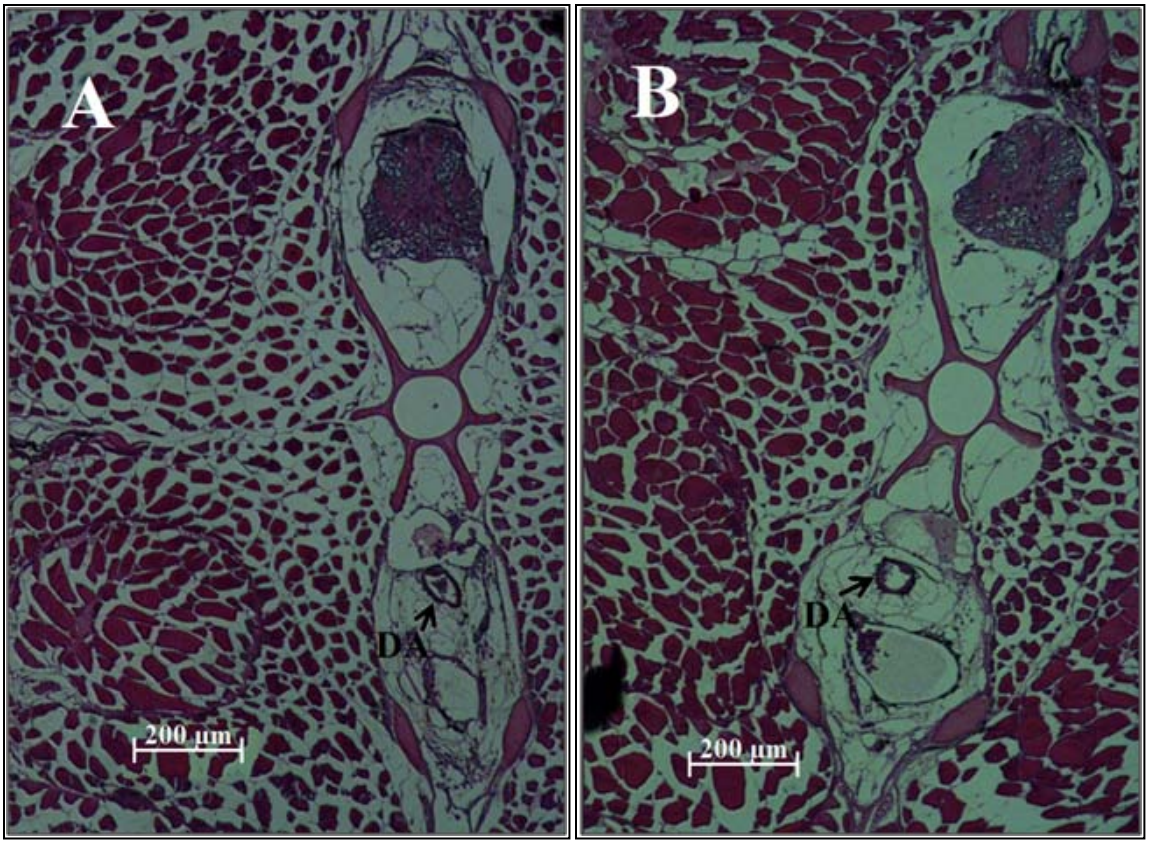

Figure 3.3 Representative images of hematoxylin/eosin stained sections of juvenile spottail shiner (*Notropis hudsonius*) dorsal aorta from reference (A; Yeoung Lake) and exposure (B; Delta Lake) sites in June 2009 at Key Lake uranium mill, Saskatchewan. DA, dorsal aorta. Images are shown at approximately 50× magnification.

fish collected from the exposure and reference lake (Table 3.4 and Figures 3.2, 3.3). No histological abnormalities were detected in cardiac or vascular structures in fish collected from either lake (Figures 3.1, 3.2, 3.3).

During fish dissections, gonads were not visible in some shiner. Of gonads that were collected, shiner were confirmed as sexually immature based on histological analysis. Only previtellogenic follicles were observed in female shiner ovaries. Of fatigued shiner in which gonads were visible, two out of nine and five out of eight were confirmed female from the exposure lake and reference lake, respectively (data not shown). In the non-fatigued group seven out of eight and three out of seven were confirmed female from the exposure lake and reference lake, respectively (data not shown).

3.3.3 Swimming performance and energy stores

No significant differences in U_{crit}, tail beat frequency or tail beat amplitude were detected between shiner collected from the two lakes (Table 3.5). Regardless of whether critical swimming speed was standardized to fish length (body length/s) or expressed as absolute velocity (cm/s) no significant swim differences were detected.

Liver glycogen was significantly greater in both fatigued (8-fold; $p<0.001$) and non-fatigued (5.4-fold; $p<0.05$) exposure site shiner compared to reference ($p<0.001$ for site factor in two-way ANOVA; 43 degrees of freedom; *F*-statistic 19.548; Figure 3.4A). Swimming also had a significant effect on liver glycogen ($p=0.049$ for swim factor; *F*-statistic 4.058), but specific pair-wise posteriori tests did not detect any significant differences. Both swimming and site significantly affected liver triglycerides ($p=0.003$ for swim factor, $p=0.009$ for site factor after two way ANOVA; *F*-statistic 9.879 for swim factor, *F*-statistic 7.618 for site factor; 40 degrees of freedom; Figure 3.4B). Liver triglycerides significantly decreased only in fish collected from

Table 3.5 Critical swimming speed (U_{crit}, body lengths/s; *n*=15-17 fish per site), tail beat frequency (hertz; *n*=15-17 fish per site) and tail amplitude (mm; *n*=15-17 fish per site) of juvenile spottail shiner (*Notropis hudsonius*) collected mid June 2009 from reference (Yeoung Lake) and exposure (Delta Lake) sites at Key Lake uranium mill, Saskatchewan.

Parameter		Reference site	Exposure site	*p*	*F*
U_{crit} (BL/s)		4.21 ± 0.35	4.50 ± 0.28	0.740	0.112
Tail beat frequency (Hz)		7.37 ± 0.34	7.88 ± 0.28	0.250	1.378
Tail amplitude (mm)	Left stroke	7.22 ± 0.87	6.52 ± 0.53	0.490	0.488
	Right stroke	7.10 ± 0.73	7.10 ± 0.48	0.999	0.001

Data expressed as mean ± standard error of the mean. 31 degrees of freedom for all parameters. Data was considered significant if $p<0.05$ using *t*-test. *p*: *p*-value; *F*: *F*-statistic.

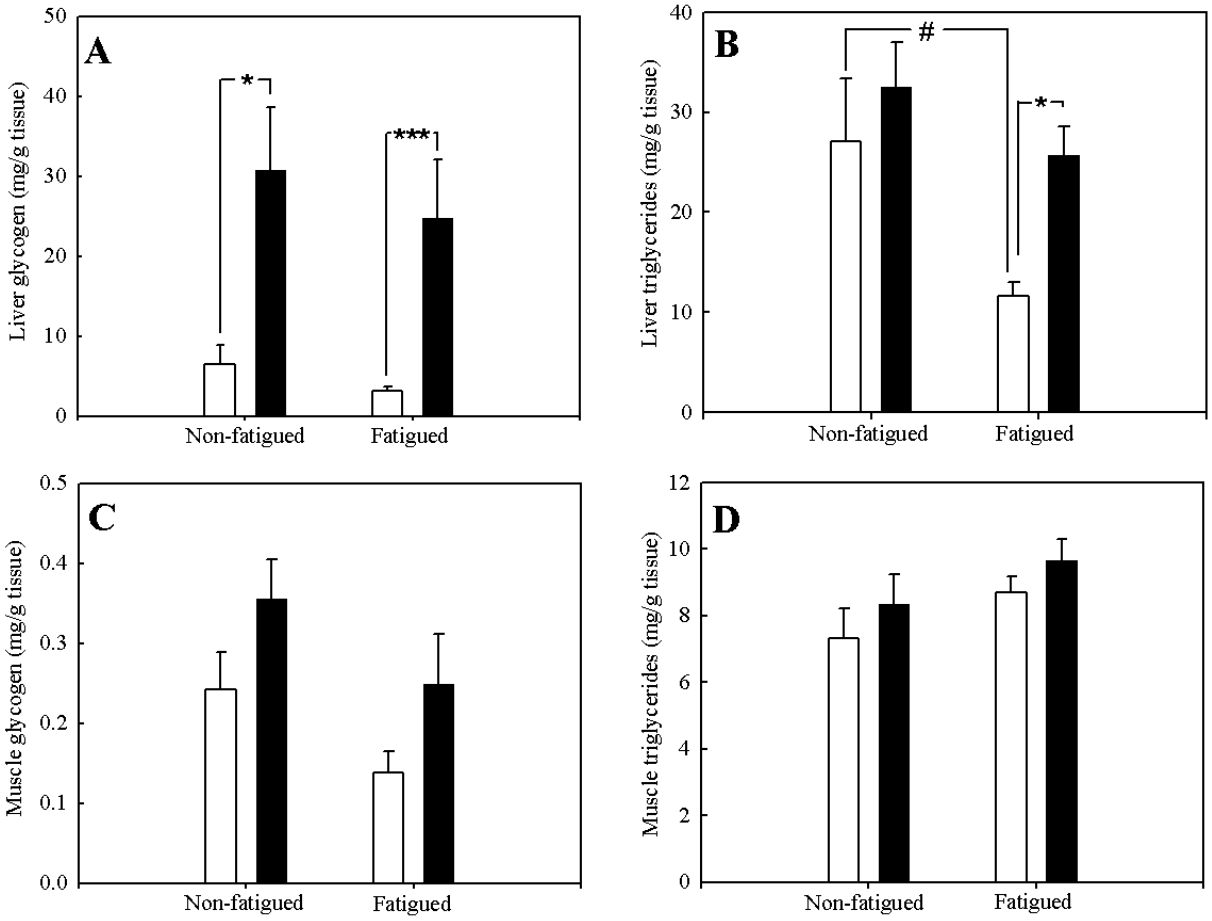

Figure 3.4 Muscle and liver glycogen and triglycerides (mg/g tissue) in juvenile spottail shiner (*Notropis hudsonius*; *n*=7-17 fish) from reference (Yeoung Lake; open bars) and exposure (Delta Lake; closed bars) sites in June 2009 at Key Lake uranium mill, Saskatchewan. For liver glycogen (1A), $p<0.001$ for site factor, $p=0.049$ for swim factor in two-way ANOVA. For liver triglycerides (1B), $p=0.009$ for site factor, $p=0.003$ for swim factor in two-way ANOVA. For muscle glycogen (1C), $p=0.031$ for site factor, $p=0.073$ for swim factor in two-way ANOVA. For muscle triglycerides (1D), $p=0.177$ for site factor, $p=0.072$ for swim factor in two-way ANOVA. Fish in "Fatigued" groups were tested in critical swimming speed tests, while fish in "Non-fatigued" groups were withheld from swim tests. #$p<0.05$ compared to non-fatigued fish from same site, *$p<0.05$, ***$p<0.001$ compared to fish from reference site in Least Square Difference posteriori tests after two-way ANOVA. Data expressed as mean ± standard error of the mean.

the reference site with swimming (p<0.05). With swimming, liver triglycerides were significantly higher in fatigued fish collected downstream of the uranium mill compared to fatigued fish collected from the reference site (p<0.05). There was a significant site effect on muscle glycogen (p=0.031 for site factor in two-way ANOVA; 19 degrees of freedom; F-statistic 5.616; Figure 3.4C) but this was not detectable in posteriori tests. Swimming did not significantly change muscle glycogen (p=0.073 for swim factor; F-statistic 1.795). Muscle triglycerides did not significantly differ with swim status (p=0.072 for swim factor in two-way ANOVA; 47 degrees of freedom; F-statistic 4.709; Figure 3.4D) or with effluent exposure (p=0.177 for site factor; F-statistic 2.861).

3.3.4 Blood parameters and enzyme activities

Swimming had a significant effect on plasma glucose (p=0.001 for swim factor in two-way ANOVA; 45 degrees of freedom; F-statistic 12.784; Figure 3.5A). Specifically, plasma glucose was significantly lower in fatigued shiner collected from the exposure lake compared to non-fatigued shiner collected from the same lake (p<0.01), but not between fatigued and non-fatigued shiner collected from the reference lake. Effluent exposure had no effect on plasma glucose within swim groups (p=0.140 for site factor in two-way ANOVA; F-statistic 2.265). Plasma lactate significantly increased in shiner collected from the reference lake with swimming (p<0.01 in Kruskal-Wallis test; 45 degrees of freedom; Figure 3.5B), but not in shiner collected from the exposure lake. Overall, plasma lactate did not differ with site. Swimming significantly affected shiner hematocrit (p=0.002 for swim factor in two-way ANOVA; 45 degrees of freedom; F-statistic 10.805; Figure 3.5C), which was significantly greater only in fatigued fish collected from the reference site compared to non-fatigued fish from the same site (p<0.01).

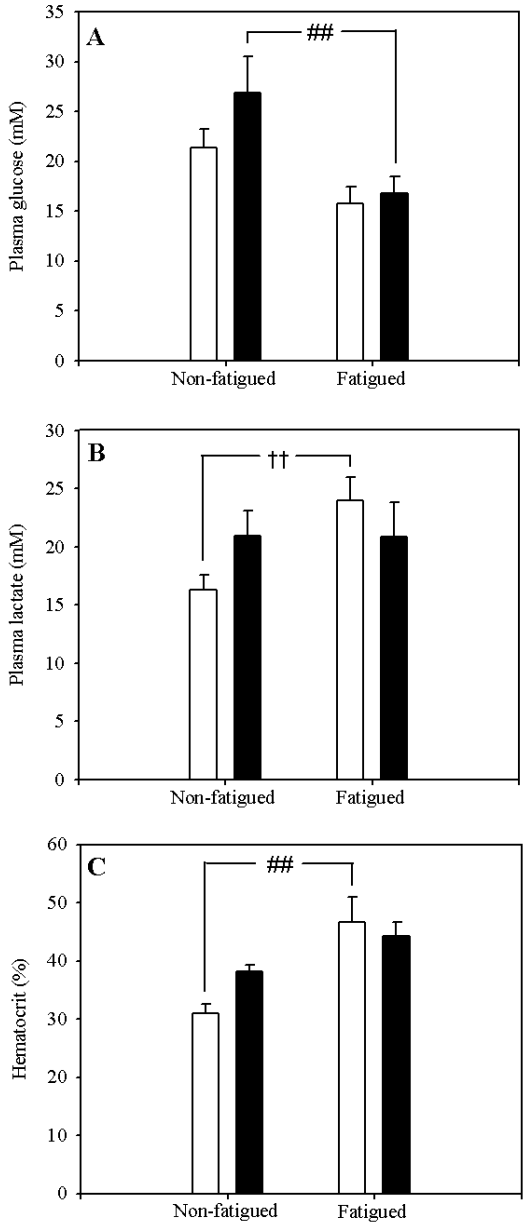

Figure 3.5 Plasma glucose (*n*=8-16 fish; 2A), plasma lactate (*n*=8-15 fish; 2B), and hematocrit (% red blood cell volume; *n*=13-17 fish; 2C) in juvenile spottail shiner (*Notropis hudsonius*) from reference (Yeoung Lake; open bars) and exposure (Delta Lake; solid bars) sites in June 2009 at Key Lake uranium mill, Saskatchewan. For plasma glucose p=0.140 for site factor, p=0.001 for swim factor in two-way ANOVA. For plasma lactate p=0.779 for site factor, p=0.156 for swim factor in two-way ANOVA. For hematocrit, p=0.456 for site factor, p=0.002 for swim factor in two-way ANOVA. Fish in "Fatigued" groups were tested in critical swimming speed tests, while fish in "Non-fatigued" groups were withheld from swim tests. †† $p<0.01$ compared to non-fatigued fish from same site in Kruskal-Wallis test; ## $p<0.01$ compared to non-fatigued fish from same site in Least Square Difference posteriori test after two-way ANOVA. Data expressed as mean ± standard error of the mean.

Effluent exposure did not significantly affect hematocrit (p=0.456 for site factor; F-statistic 0.567).

There were no significant differences in muscle enzyme activity between fatigued and non-fatigued shiner collected from the same lake (data not shown). As a result, activity data for all shiner collected from the same lake were combined for each enzyme. Muscle HOAD activity was significantly greater in shiner collected from the exposure lake (0.54 ± 0.05 IU/g tissue; p<0.01; Table 3.6) compared to reference (0.34 ± 0.03 IU/g tissue), but no difference was observed in muscle CS activity between lakes.

Table 3.6 Citrate synthase (CS; *n*=22-24 fish per site) and β–hydroxyacyl coenzyme A dehydrogenase (HOAD; *n*=16-19 fish per site) activities (International Units/g tissue) in juvenile spottail shiner (*Notropis hudsonius*) dorsal muscle collected mid-June 2009 from reference (Yeoung Lake) and exposure (Delta Lake) sites at Key Lake uranium mill, Saskatchewan.

	Reference site	Exposure site	*p*	d.f.	*F*
CS	3.31 ± 0.18	3.10 ± 0.16	0.377	45	0.794
HOAD	0.34 ± 0.03	0.54 ± 0.05	0.004	34	7.080

Data expressed as mean ± standard error of the mean. Data was considered significant if $p<0.05$ using *t*-test. *p*: *p*-value; d.f.: degrees of freedom; *F*: *F*-statistic.

3.4 Discussion

3.4.1 Energy homeostasis

Few studies have investigated energy homeostasis and swimming ability in wild fish exposed to complex industrial effluents, and this study is the first in fish inhabiting aquatic systems affected by uranium milling effluent. The major finding of the present study was that despite significant alterations in trace element body burdens, carbohydrate and triglyceride homeostasis, there were no apparent effects on swimming performance. Enlarged ventricle relative to body size and elevated body burdens of some trace elements (boron, molybdenum, selenium, and thallium) did not affect tail beat frequency, tail amplitude, or U_{crit} in shiner collected from the exposure lake compared to shiner collected from the reference lake. Shiner collected from the exposure lake had significantly higher HOAD muscle activity, but it is unclear how this might relate to altered hepatic triglyceride metabolism in fatigued shiner from the same lake. Hematocrit and plasma lactate increased only in fish collected from the reference lake with swimming, further indicating the presence of metabolic alterations in fish collected downstream of the Key Lake uranium mill.

Glycogen homeostasis was significantly altered in shiner collected from the exposure lake. Similar to earlier studies in fish collected downstream of the Key Lake uranium mill, elevated hepatic glycogen stores were observed in these shiner (Kelly and Janz, 2008). However, previous studies evaluating wild fish from other metal-contaminated systems report inconsistent effects on glycogen stores and it is uncertain if these effects are environmentally or toxicologically related (Levesque et al., 2002; Weber et al., 2008). It is interesting to note that shiner collected from the exposure lake had reduced liver size despite significantly greater glycogen concentration compared to reference. Exposure to other environmental contaminants

has been reported to cause variable effects on fish liver size, in some cases causing hypertrophy as a result of increased metabolic demand from augmented detoxification (Bhattacharya et al., 2007; Sun et al., 2007; Khan, 2010). However, in the present study smaller livers were observed in shiner downstream of the Key Lake uranium mill compared to reference, which supports previous studies reporting decreased liver size in spottail shiner from the same lake (Golder Associates, 2008). The factors influencing liver size and glycogen storage in fish downstream of the Key Lake uranium mill remain unclear. Although reduced liver size is consistently reported in these shiner, which could be indicative of some toxic effect of effluent exposure, elevated liver glycogen suggests otherwise. Overall, shiner downstream of the Key Lake uranium mill have greater total glycogen stores than fish collected from the reference lake, despite lower liver size, and further research is needed to better understand relationships between energy stores and liver size in this species.

Shiner collected downstream of the Key Lake uranium mill also exhibited significant alterations involving triglyceride metabolism. While elevated liver triglycerides have been reported in juvenile pike and burbot (*Lota lota*) collected downstream of the Key Lake uranium mill (Bennett and Janz, 2007; Kelly and Janz, 2008), in the present study liver and muscle triglycerides in non-fatigued shiner were comparable between lakes. Interestingly, only shiner collected from the reference lake had significantly lower liver triglycerides after swimming. This suggests lipids mobilized from hepatic stores play an important role in spottail shiner exercise metabolism. It is possible that fatigued shiner collected from the exposure site mobilized triglycerides from other lipid storage sites (*e.g.* visceral stores), but few *in vivo* studies document which lipid stores are mobilized during activity and the factors that influence this (Moyes and West, 1995). Alternatively, these fish may have utilized plasma glucose in place of hepatic

triglycerides, as plasma concentrations decreased only in shiner collected from the exposure lake with swimming. While aerobic muscle can use circulating glucose as a fuel substrate during aerobic exercise, previous studies suggest this tissue preferentially metabolizes lipids (Webb, 1971a; West et al., 1993). Instead, glucose derived from intramuscular glycogen is a preferred energy substrate in anaerobic muscle metabolism, which results in lactate production (Moyes and West, 1995). Overall, effluent exposure may alter fuel substrate preference during aerobic exercise, but further research is required investigating factors that influence lipid metabolism in fish.

Altered triglyceride metabolism could be related to changes in tissue-level enzyme activity. Citrate synthase and HOAD enzyme activities determined in the current study are consistent with teleost muscle values obtained in other studies (Pelletier et al., 1994; Rajotte and Couture, 2002). Comparable CS activity in shiner collected from both lakes suggests intramuscular ability to aerobically metabolize fuel substrates for ATP production is similar between lakes. This conclusion is based on the fact that CS is a rate-limiting enzyme involved in the Krebs cycle and is a general indicator of aerobic capacity (Rajotte and Couture, 2002; Lemos et al., 2003). The mitochondrial enzyme HOAD is involved in triglyceride β-oxidation and is considered an indicator of aerobic lipid catabolism (Londraville and Duvall, 2002; Rajotte and Couture, 2002). In the present study muscle HOAD activity was elevated in shiner collected from the exposure lake compared to the reference lake. Elevated muscle HOAD activity may be a compensatory mechanism for the observed reduction in liver triglyceride metabolism in shiner collected downstream of the uranium milling operation. Interestingly, significantly reduced muscle HOAD activity was reported in perch from a highly metal-contaminated lake and this coincided with lower U_{crit} (Rajotte and Couture, 2002), while the current study found no change

in U_{crit} in fish with higher HOAD activity. Thus, muscle HOAD activity is not clearly linked to swimming endurance, but further research is required to elucidate the relationship between metabolic enzyme activities and aerobic exercise metabolism in fish.

Altered hematocrit, plasma lactate and glucose in shiner collected from the exposure lake could be attributed to elevated water hardness and/or overall ionic strength compared to the reference lake, but may also signify stress-related effects. Soft water-acclimated freshwater fish maintain osmoregularity via gill chloride cell proliferation, but this adaptation can increase blood-to-water diffusion distance and limit blood-gas exchange (Greco et al., 1996; Dussault et al., 2008). Consequently, previous studies reported elevated hematocrit, blood lactate and glucose, alongside lower swim endurance, in fish acclimated and exercised in soft water (Kieffer et al., 2002; Dussault et al., 2008). In the present study, this physiological response was confirmed in fish from the reference lake (which had substantially lower conductivity and hardness compared to the exposure lake), but swimming endurance was still similar between sites. Plasma lactate remained constant between fatigued and non-fatigued fish from the exposure site, which may be due to impaired lactate dehydrogenase enzyme activity (Rajotte and Couture, 2002) or elevated lactate recovery during swimming (Moyes and West, 1995). On the other hand, lactate and hematocrit in shiner collected from the exposure lake may have failed to further increase with swimming as these endpoints appeared elevated prior to swimming compared to fish collected from the reference lake, although this difference was not significant. Plasma glucose also showed a trend to be elevated in non-fatigued fish collected from the exposure site compared to non-fatigued fish collected from the reference lake. The changes observed in the current study are consistent with known metabolic alterations caused by

physiological stress, suggesting that activation of acute physiological stress pathways may be present in fish downstream of the uranium milling operation at Key Lake.

3.4.2 Swimming performance

Fish collected from both lakes had comparable U_{crit}, which parallels similar tissue aerobic capacity at the biochemical level (*i.e*. CS activity) in fish from both sites. Up to approximately 80% U_{crit} is based on aerobic metabolism (Webb, 1971a). It follows that CS plays a key role in generating ATP to working tissue, such that CS activity levels could dictate tissue metabolic capacity, thus influencing swimming ability. Swimming ability or locomotory function seems to parallel both aerobic and anaerobic CS muscle activity (Torres and Somero, 1988; Gibb and Dickson, 2002; Rajotte and Couture, 2002). In both fatigued and non-fatigued fish major energy store differences between sites were observed in liver but not muscle. Swimming performance tests can activate metabolic pathways related to both exercise and the physiological stress response, particularly those involving catecholamine mobilization (Jobling, 1994). During an acute stress stimulus catecholamines and cortisol target hepatic energy stores, mobilizing triglyceride stores and stimulating glycogenolysis and gluconeogenesis to increase circulatory glucose. However, most energy to fuel swimming will be derived from intramuscular stores (van den Thillart, 1986; Moyes and West, 1995). It is plausible these stores could also be utilized during a stress response. However, it seems intramuscular triglyceride or glycogen reserves are not mobilised by acute stress response hormones to the same degree as hepatic stores and therefore change relatively little during the stress response (Fabbri et al., 1998; Mommsen et al., 1999). Therefore, in the present study similar U_{crit} between sites could be attributed to similar intramuscular metabolism, which is supported by comparable CS activity and lack of change in intramuscular energy stores after swimming. However, shiner collected from the exposure lake

had reduced hepatic triglyceride mobilization during swimming compared to reference fish, which (in addition to altered plasma glucose, lactate and hematocrit) could indicate an effluent-related alteration in the physiological stress response. This may be the case in other studies that report no change in swimming performance in contaminant exposed wild fish despite alterations in metabolism or the physiological stress response (Taylor et al., 2004; McKenzie et al., 2007).

3.4.3 Water chemistry and trace metal effects on energy homeostasis and swimming performance

Water chemistry differences between lakes (due to discharged mill effluent) could have contributed to energy store alterations observed in shiner downstream of the Key Lake uranium mill. Water chemistry values reported here are generally consistent with previous data for these lakes (Bennett and Janz, 2007; Golder Associates, 2008; Kelly and Janz, 2008). Water bodies downstream of the effluent discharge point are consistently characterized by elevated total dissolved solids, salinity, conductivity, ammonia, and hardness compared to reference lakes (Pyle et al., 2001; Bennett and Janz, 2007; Kelly and Janz, 2008; Muscatello et al., 2008). Nitrogen (as nitrate and ammonia) from discharged effluent could increase food web productivity in systems downstream of the Key Lake mill, contributing to elevated stores in resident organisms (Elser et al., 1990). However, past studies fail to support this hypothesis (Kelly and Janz, 2008). Instead, an environmental enrichment effect could be due to the high ion content of the mill effluent. Average 2007 water conductivity in four reference lakes near the mill (including the reference lake used for the present study) was 18 ± 1 μS/cm, compared to 562 μS/cm in the presently investigated exposure lake (Golder Associates, 2008). Elevated conductivity could bring resident water closer to fish homeostatic neutrality. This phenomenon would increase the energetic favorability of ionoregulation, potentially leading to the allocation

of greater energy to storage and growth in shiner collected from the exposure lake (Shuter et al., 1989). Conversely, resident shiner from reference lakes would have to allocate a greater percentage of energy reserves towards ionoregulation, thus decreasing overall energy stores.

Trace element-related gross morphological effects were absent in shiner collected downstream of the uranium mill. In general, the trace element body burden results in the present study support earlier reports of similar elevated metals and metalloids in exposure lake water and sediment (Pyle et al., 2001; Golder Associates, 2008). Extensive work has occurred at the Key lake uranium mill documenting the bioaccumulative and teratogenic nature of the effluent discharged into the receiving environment (Muscatello et al., 2006; Muscatello et al., 2008; Wiramanaden et al., 2010). In one study pike from the same exposure lake had approximately 10-fold greater muscle selenium concentrations than reference lake pike, and generated larvae with significantly elevated incidences of edema and gross deformities (Muscatello et al., 2006). However, in the current study shiner collected from the exposure lake had whole body selenium concentrations 7.8-fold greater than reference, but no morphological deformities were observed. This is supported by an earlier study in which no body deformities were observed out of 68 shiner collected from the same exposure lake (Golder Associates, 2008). In the same study mean whole body selenium concentration in shiner from the exposure lake was 4.7 ± 0.5 μg/g wet weight (corresponding to 18.1 μg/g dry weight based on 74.04% moisture content; Golder Associates, 2008). It is plausible that spottail shiner are less sensitive to selenium toxicity than other northern fish species. However, shiner collected from the exposure lake in the present study had significantly lower mass, length, and liver size compared to shiner collected from the reference lake. Although reduced size could be attributed to trace metal toxicity, other factors

must be considered, including differences in seasonal lake temperature, water chemistry, sediment composition, or trophic structure.

Earlier studies report negative effects of some trace elements (aluminum, copper, cadmium, zinc) on swimming performance (Waiwood and Beamish, 1978; Wilson and Wood, 1992; Beaumont et al., 1995; McGeer et al., 2000). However, in the present study, elevated body burdens of certain trace elements (boron, molybdenum, selenium, thallium) did not translate into altered swimming since U_{crit} was unchanged in shiner collected from the exposure lake. Furthermore, since swim motion (tail beat frequency and amplitude) was similar between sites, this suggests that there were no spinal or related morphological effects caused by the observed elevated trace element body burdens. Conversely, elevated trace elements could in part be responsible for greater ventricle diameter observed in shiner collected from the exposure lake. As discussed earlier, selenium is known to cause cardiovascular terata in larval fish (Holm et al., 2005; Muscatello et al., 2006), but it is unclear if these effects persist in juvenile and adult stages of wild fish. It has been demonstrated that ventricular morphology significantly affects cardiovascular ability, and thus swim performance (Claireaux et al., 2005). Given the importance of cardiovascular function on swim endurance, as well as numerous studies demonstrating negative effects of trace metal exposure on swimming performance, it is interesting that in the present study U_{crit} was similar between lakes. Overall, further investigation is warranted to elucidate the threshold for trace element body burdens and degree of metabolic alteration needed, if any, for swimming ability to be altered in spottail shiner.

3.5 Conclusions

Swimming performance was similar in shiner despite significant cardiovascular and metabolic effects in fish collected downstream of the uranium mill. Shiner collected from the

exposure lake had elevated hepatic glycogen stores, which could be related to a more homeostatically neutral (higher ion) environment or an alteration in the physiological stress response. Fish collected from the exposure lake had reduced access to liver triglycerides during swimming, but HOAD activity was greater in muscle compared to fish collected from the reference lake. Shiner collected from the exposure lake failed to increase plasma lactate and hematocrit, but decreased plasma glucose during U_{crit} tests. Despite metabolic alterations, muscle CS activity, intramuscular energy stores, tail beat frequency and amplitude were all comparable between sites, supporting the conclusion that there was similar swimming ability in fish between sites. Future studies will investigate the role of tissue enzyme activity on energy substrate metabolism, and attempt to further characterize the physiological effects of complex contaminant mixtures on wild fish populations.

CHAPTER 4
4.0 SWIMMING PERFORMANCE AND ENERGY HOMEOSTASIS IN JUVENILE LABORATORY RAISED FATHEAD MINNOW (*PIMEPHALES PROMELAS*) EXPOSED TO URANIUM MILL EFFLUENT

4.1 Introduction

Environmental contaminants can alter biochemical and physiological processes that play crucial roles in fish survival. Integrated physiological traits, such as swimming performance and metabolic status, can provide greater insight on contaminant effects in fish than commonly used acute endpoints (Rajotte and Couture, 2002; McKenzie et al., 2007). Indeed, acute tests generally fail to address behavioural or physiological changes that could subtly compromise an organism's ability to function normally in its environment (Scott and Sloman, 2004). Swimming is considered a complex integrated behaviour that involves several levels of organismal processes. If impaired, reduced swimming ability could significantly affect survivability as fish depend on swimming for activities such as feeding, predator evasion, migration and mating (Beaumont et al., 1995; Drucker, 1996; Plaut, 2001). Critical swimming speed (U_{crit}) is a commonly employed incremental velocity swim test reflective of endurance and maximal swim speed (Brett, 1964). It is estimated that up to 80% U_{crit} occurs via aerobic metabolism, and this swim test has become a standard measure of aerobic performance (Webb, 1971a). Furthermore, U_{crit} can provide an ecologically relevant assessment of swimming ability (reviewed by Plaut, 2001), and has been shown to be a sensitive measure of exposure to many environmental contaminants, including ammonia (reviewed by McKenzie et al., 2003), dissolved metals (Wilson and Wood, 1992; Beaumont et al., 1995; Alsop et al., 1999; McGeer et al., 2000; Rajotte and Couture, 2002; Taylor et al., 2004) and complex mixtures such as coal ash (Hopkins et al., 2003), crude oil fractions (Kennedy and Farrell, 2006), or urban river systems (McKenzie et al., 2007).

Some contaminants have the ability to cause subtle effects in fish that may significantly impair physical swimming and/or cardiovascular ability. Most notably, certain studies have demonstrated that selenium causes morphological and cardiovascular terata in maternally exposed fish larvae (Holm et al., 2005; Muscatello et al., 2006). Morphological deformities could impair swim motion characteristics, such as tail beat frequency or tail beat amplitude, while cardiovascular abnormalities could impair oxygen delivery to aerobic tissues.

Swimming performance is closely tied to intermediary metabolism and energy homeostasis. Burst swimming requires white muscle activity, from which energy is chiefly derived from intramuscular glycolysis via anaerobic metabolic pathways (Webb, 1971a; Hammer, 1995). In contrast, sustained activity or aerobic swimming recruits red muscle up to 80% U_{crit}, and is primarily fuelled by triglyceride β-oxidation (Webb, 1971a; Moyes and West, 1995). Measuring the activity of β–hydroxyacyl coenzyme A dehydrogenase (HOAD), a rate-limiting mitochondrial enzyme involved in triglyceride β–oxidation, is considered an indicator of lipid metabolism. Trained rainbow trout have elevated skeletal muscle HOAD activity compared to untrained trout, indicating that elevated lipid metabolism positively correlates with U_{crit} (Farrell et al., 1991). During activity, oxidative phosphorylation and oxygen availability (via the circulatory system) generally dictate aerobic scope (Weber and Haman, 1996). Thus, it follows that citrate synthase (CS), a rate-limiting mitochondrial enzyme involved in the citric acid cycle, is commonly used as an indicator of tissue aerobic scope. For example, muscle CS activity was positively correlated with sustainable swimming speed in juvenile Kawakawa tuna (*Euthynnus affinis*; Gibb and Dickson, 2002). Furthermore, more active fish species tend to have higher CS muscle activity than less active species (reviewed by Dickson, 1995).

Some studies report intermediary metabolic effects in fish inhabiting metal-contaminated environments. For example, wild yellow perch (*Perca flavescens*) from metal-contaminated lakes were reported to have decreased muscle enzyme activities (CS, HOAD, lactate dehydrogenase; Rajotte and Couture, 2002) and altered seasonal energetic stores (Levesque et al., 2002). Recently, the energy stores of certain fish species downstream of the Key Lake uranium mill (Saskatchewan, Canada) have been investigated, as this mill discharges effluent known to contain arsenic, molybdenum, nickel, selenium, uranium, thallium, ammonia and organics into the local water system. Some fish species downstream of the mill have an altered energetic status that includes increased triglyceride and glycogen stores (Bennett and Janz, 2007; Kelly and Janz, 2008). However, it is unclear if the metabolic and swimming performance effects observed in other fish species from metal-contaminated areas are present in fish exposed to Key Lake uranium mill effluent.

The objective of this study was to investigate the potential swimming and energy homeostatic effects in laboratory raised juvenile fathead minnow (FHM; *Pimephales promelas*) exposed to diluted Key Lake uranium mill effluent. Although the FHM is used extensively in North American aquatic research, relatively little is known about the effects of contaminant exposure on swimming performance and intermediary metabolism on this species (Ankley and Villeneuve, 2006). In this study FHM larvae obtained from effluent exposed adult breeding pairs were raised to 60 day post hatch in either 5% effluent or dechlorinated municipal water. Swimming ability (U_{crit}, tail beat frequency, tail stroke amplitude) was measured in fish, followed by morphometric endpoints (mass, fork length, condition factor), cardiovascular morphological endpoints (bulbus arteriosus, ventricle, dorsal aorta diameter), whole body energy store concentrations (glycogen, triglycerides) and metabolic enzyme activities (CS, HOAD).

4.2 Materials and Methods

4.2.1 Parental exposure and breeding

Fathead minnow (approximately 6 months old) were obtained from Osage Catfisheries, Inc. (Osage Beach, MO). Fish were kept at 20°C with a light/dark cycle of 16/8hr at all life stages. All fish in the control treatment group were kept in an aerated 65% reverse osmosis municipal water/35% dechlorinated municipal water mixture throughout the experiment. For the duration of the study fresh effluent was shipped from the Key Lake uranium mill to Saskatoon, SK every 4-5 days. All adult breeding pairs and subsequent larvae and juveniles in the exposure treatment were maintained in an aerated 5% mill effluent/95% control water mixture. This concentration was selected as it generally reflects the effluent concentration within Delta Lake (Saskatchewan, Canada), a lake approximately 10 km downstream of the Key Lake uranium mill that contains sustainable fish populations and is subjected to ongoing environmental effects monitoring (Muscatello et al., 2006; Bennett and Janz, 2007; Keller, 2007; Golder Associates, 2008).

Adult breeding pairs were exposed to 5% effluent or control water for 17 days. During this period both groups were fed untreated frozen blood worms twice daily (San Francisco Bay Brand, Newark, CA). Adult breeding pairs were then placed in trophic-transfer artificial stream systems using the same exposure treatments. Chironomids were co-cultured in artificial streams so that adults had both dietary and aqueous effluent exposure. Each artificial stream system was supplied by an 85 L reservoir tank with a turnover rate of one full reservoir volume per day of respective treatment. All other system details, including stream flow rates, volumes, and mechanical setup were as described previously (Rickwood et al., 2006). At all life stages fish were treated in accordance with protocols approved by the University of Saskatchewan Animal

Research Ethics Board and the Canadian Council on Animal Care guidelines on experimental animal care and use.

4.2.2 Larval husbandry

Eggs from adult breeding pairs were collected 31-38 days after the onset of the exposure period and placed in mesh-bottom polyvinyl chloride cups submerged in respective treatments. Larvae were raised under the same light and temperature conditions as adults. Starting at 3 days post hatch (dph), larvae were fed freshly hatched brine shrimp (*Artemia salina*) twice daily until the conclusion of the experiment. Brine shrimp were hatched in the same water treatments as respective larvae. Between 5-10 dph, larvae were transferred to submerged mesh baskets in separate continuous flow-through systems containing either the exposure or control treatment. Fish were maintained in this system until 60 dph. System flow rate equalled one full tank (12 L) turnover per day with two replicate tanks for each treatment. Tanks were cleaned daily of excess fecal matter, mortalities (if necessary) and brine shrimp. Ammonia and pH were measured every three days using test kits (Aquarium Pharmaceuticals, Mars Fishcare., Chalfont, PA). Conductivity, total dissolved solids and salinity were measured every 20 days using a multi-parameter YSI probe (6 series - YSI Inc., Yellow Springs, OH).

4.2.3 Swimming performance

Fish were left unfed for eight hours before swimming tests to obtain a postabsorptive state (Niimi and Beamish, 1974; Farlinger and Beamish, 1977). Critical swimming speed was evaluated in individual fish using a LoligoSystems Mini Swim Tunnel (LoligoSystems, Tjele, Denmark). The swim tunnel apparatus was filled with approximately 50 L of circulating aerated 5% effluent treatment or control water, and temperature was maintained at 20°C. Fish were allowed to acclimate in the swim chamber for 45 minutes at low flow (approximately 1 body

length/s). During experiments water velocity was increased stepwise in 3-minute increments by 1.5 cm/s (*i.e.* velocity increased slightly less than 1 body length/increment) until fatigue. This protocol was directly adapted from previous studies (Kaufmann, 1990; Dussault et al., 2008), which were modified to accommodate early life stage and/or small-bodied fish in small swim tunnels. Critical swimming speed was calculated as: $U_{crit} = V_p+((t_f / t_i)\times V_i)$, where V_i is the velocity increase per increment, V_p is the final velocity swam, t_i is the increment time length and t_f is the duration of the last velocity increment until fatigue.

Swim motion was recorded concurrently with a high speed camera (250 frames/second) to enable measurement of tail beat frequency and tail beat amplitude using Midas 2.0 imaging software. Both endpoints were calculated at the highest completed velocity interval during U_{crit} testing. Tail beat frequency was analyzed directly using Adobe Premiere Elements 2.0, while video frames for tail amplitude analysis were selected using this software and then analysed using Image-Pro 6.0. Tail beat amplitude was calculated as the maximum deviation distance of the most posterior point of the caudal peduncle from the longitudinal body line, which was averaged over three separate tail beat cycles for each fish.

A second group of fish from the same cohort from each treatment was withheld from swimming experiments, but subjected to the same handling as swam fish. This group without swim testing was considered non-fatigued and metabolic endpoints were compared to fatigued fish.

4.2.4 Morphometrics and histology

Fish were anaesthetized using an overdose of Aquacalm (5 mg metomidate hydrochloride/L; Syndel Laboratories Ltd., Qualicum Beach, BC) immediately after swim performance experiments. After fork length was recorded, a sub-set of whole fish (*n*=8-13 fish

from each fatigued or non-fatigued group per treatment) was stored at -80°C until subsequent biochemical analyses. Condition factor was calculated as: [(100×weight)/(length^3)]. The remaining fish (*n*=6-8 fish per treatment, mixed fatigued and non-fatigued) were preserved in Bouin's fixative for 36 hours and stored in 70% ethanol until histological preparation. For histological analysis paraffin-embedded whole fish were sectioned serially in 5 μm cross sections, which were eosin/hematoxylin stained. Bulbus arteriosus and ventricle diameter were assessed at 50× magnification, and dorsal aorta at 200× magnification, using an Axio Observer.Z1 inverted microscope (Carl Zeiss MicroImaging, LLC, Goettingen, Germany). Bulbus arteriosus muscle diameter and ventricle diameter were measured at the maximum chamber width, averaged over three consecutive sections. Dorsal aorta inner diameter was measured at the point immediately posterior of the anus and averaged over three consecutive sections for each fish.

4.2.5 Trace element body burdens

For whole body trace element analysis, FHM (*n*=5 non-fatigued fish per treatment) were freeze dried for 24-48 hours. All fish from each treatment were pooled and ground using a porcelain mortar and pestle for subsequent tissue digestion. Percent moisture content was calculated as: [100×(wet mass-dry mass)/(wet mass)]. For each pooled sample, approximately 0.05 g was cold digested in Teflon vessels using 5 ml of ultra-pure nitric acid and 1.5 ml of hydrogen peroxide (30%; Suprapur, EMD Chemicals, Gibbstown, NJ). Digested samples were evaporated in Teflon vessels at approximately 65˚C and then reconstituted in 5 ml of 2% nitric acid. Prior to instrumental analysis reconstituted samples were syringe filtered using 0.45 μm polyethersulfone filters. Trace element concentrations in pooled whole body fish (μg/g dry weight) were evaluated using inductively coupled plasma-mass spectrometry (ICP-MS) at the

Toxicology Centre (University of Saskatchewan, Saskatoon, SK) with a certified reference material (TORT-2, lobster hepatopancreas) obtained from the National Research Council of Canada (Ottawa, ON) as described previously (Phibbs et al., 2011).

4.2.6 Laboratory analyses

4.2.6.1 Energy stores

All reagents were purchased from Sigma Aldrich (Sigma Aldrich Canada Ltd., Oakville, ON) unless otherwise specified. Whole body triglyceride content was determined in individual fish as previously described (Weber et al., 2003). Glycogen was determined using a colourimetric assay as previously described with slight modification (Weber et al., 2008). Briefly, incubation with amylase was adjusted to 60°C for 2 hours to ensure complete glycogen digestion.

4.2.6.2 Enzyme activities

Enzyme activities (CS and HOAD) in individual whole fish were determined. Fish were thawed on ice, weighed, diluted 4× with homogenizing buffer (100 mM Tris, 3 mM EDTA, 2 mM $MgCl_2$, 1 mM reduced glutathione, 0.1% peroxide- and carbonyl-free Triton-X100; pH 7.4), minced with scissors and homogenized on ice (5×15s) using a PowerGen Model 125 homogenizer (Fisher Scientific Company, Ottawa, ON). Immediately prior to enzyme assays all homogenates were centrifuged at 1000×g at 4°C for 5 minutes to remove insoluble tissues.

Citrate synthase activity was determined in individual whole fish homogenates using a modified kit (Sigma Aldrich). Specific activity was determined by assaying each sample in the presence or absence of palmitoyl coenzyme A (100 mM), a CS specific inhibitor, in triplicate. Enzyme activity was measured at 412 nm at 22°C over 1.5 minutes. Specific activity was determined for each sample by subtracting non-specific absorbance from total absorbance.

Preliminary experiments determined optimal β–hydroxyacyl coenzyme A dehydrogenase (HOAD) activity conditions based on protocols of Rajotte and Couture (2002) and Pelletier et al. (1994). Activity was determined in whole fish homogenates by measuring the oxidation of NADH at 340 nm under the following conditions: 100 mM Tris, 1 mM EDTA, 1 mM KCN, 0.16 mM NADH, pH 7.4. Samples were assayed in triplicate in the presence or absence of acetoacetyl coenzyme A (0.1 mM). Plates were read at 340 nm at 25°C over 10 minutes. Specific activity was determined by subtracting the linear absorbance change in vehicle control wells (endogenous activity) from wells containing acetoacetyl coenzyme A. Whole body protein concentration was determined in a protein assay (Biorad, Hercules, CA), using bovine serum albumin as the standard.

4.2.7 Statistical analyses

Data were tested with Shapiro-Wilk normality test and Bartlett's test for homogeneity of variance. Data that failed these tests were log10 transformed to achieve normality and homogeneity of variance. Significant differences in fork length, body mass, U_{crit} (body lengths/s), absolute U_{crit} (cm/s), tail beat frequency and amplitude, condition factor and cardiovascular endpoints (standardized to mass) were detected using a *t*-test. Two-way analysis of variance (ANOVA) was used to analyze swim and metabolism data with treatment (5% effluent vs. control) and swim status (fatigued vs. non-fatigued) as factors, followed by Least Square Difference posteriori tests. There were no interactions between factors for all two-way ANOVA analyses. Results were considered significant if $p<0.05$. Data are reported as mean ± standard error of the mean (SEM).

4.3 Results

For water chemistry, there was no difference in pH or temperature between treatments (n=17 measurements per treatment for pH; n=3 measurements per treatment for temperature; Table 4.1). Conductivity (p<0.001), total dissolved solids (p<0.001), salinity (p<0.001) were all significantly higher in the 5% effluent treatment compared to control (n=3 measurements per treatment; Table 4.1). Ammonia was consistently elevated in the exposure tanks (p<0.01; n=17 measurements per treatment) compared to control (Table 1).

Due to their small size FHM were pooled to perform trace element analysis and for this reason statistical analysis was not possible (Table 4.2). As a result, trace element body burdens in fish from the 5% effluent treatment were considered different if values were 50% higher or 50% lower than control fish. Using these criteria the arsenic body burden in effluent exposed fish was greater compared to control treatment fish, while chromium, iron, lead, manganese, molybdenum, nickel, silver, tin, and uranium body burdens were lower. Average moisture content of the two pooled samples was 88.4%.

While fish mass was not significantly different between treatments at 60 dph, fork length was significantly lower in effluent exposed fish (p<0.05) compared to control fish (Table 4.3). Condition factor was significantly greater in juvenile fish from the exposure (p<0.01) compared to the control treatment (Table 4.3). Cumulative mortality was similar (71.8 – 73.3%) between treatments but statistics could not be performed with only two replicate tanks per treatment. Highest mortality rates occurred between 7 and 15 dph in all tank replicates (data not shown). Histologically, ventricle diameter (n=8 fish per treatment; Figure 4.1), bulbus arteriosus muscle diameter (n=8 fish per treatment; Figure 4.2), heart and dorsal aorta inner diameter (n=6-8 fish per treatment; Figure 4.3) were not statistically different between the control and exposure

Table 4.1 Water chemistry parameters for municipal water and 5% Key Lake uranium mill (Saskatchewan, Canada) effluent treatments.

Parameter	Municipal water	5% effluent
Temperature (°C)	20.6 ± 0.1	19.8 ± 0.4
pH	7.92 ± 0.07	7.88 ± 0.04
Conductivity (μS/cm)	339 ± 6	510 ± 13***
Total dissolved solids (mg/L)	0.25 ± 0.01	0.37 ± 0.01***
Salinity (mg/L)	0.18 ± 0.01	0.27 ± 0.01***
Ammonia (mg/L)	0.06 ± 0.03	0.44 ± 0.09**
Hardness (mg/L)[†]	47.3 ± 2.5	146.7 ± 18.6
Sulphate (mg/L)[†]	34.3 ± 0.6	140.0 ± 20.0
Carbonate (mg/L)[†]	0.5 ± 0.1	0.5 ± 0.1

Data expressed as mean ± standard error of the mean.
**$p<0.01$, ***$p<0.001$ compared to municipal water treatment using *t*-test.
Values averaged from two replicate tanks per treatment. Ammonia and pH measured approximately every three to four days ($n=17$ measurements per treatment); temperature, conductivity, total dissolved solids and salinity averaged from measurements taken on days 20, 40 and 60 ($n=3$ measurements per treatment).
[†]Hardness, sulphate and carbonate values obtained from Driessnack et al., unpublished.

Table 4.2 Pooled trace element body burdens (µg/g dry weight; *n*=1 replicate per treatment, each replicate consisting of *n*=5 pooled 60 dph fish) in fathead minnow (*Pimephales promelas*) from municipal water and 5% Key Lake uranium mill (Saskatchewan, Canada) effluent treatments.

Analyte	Municipal water	5% effluent
Aluminum	19.24	27.45
Arsenic	0.94	1.52
Barium	4.92	3.95
Cadmium	0.09	BDL
Chromium	36.38	11.65
Cobalt	0.08	0.10
Copper	7.55	8.25
Iron	273.52	130.92
Lead	0.43	0.10
Manganese	5.34	2.37
Mercury	0.16	0.14
Molybdenum	3.11	1.30
Nickel	1.02	0.44
Selenium	1.09	1.17
Silver	0.15	0.01
Strontium	55.47	52.86
Tin	1.25	0.46
Titanium	40.09	32.92
Thallium	<0.01	0.01
Uranium	0.02	0.01
Vanadium	0.13	0.15
Zinc	98.67	99.69

BDL: Below detection limits

Table 4.3 Morphometric data for 60 day post hatch fathead minnow (*Pimephales promelas*) from municipal water and 5% effluent Key Lake uranium mill (Saskatchewan, Canada) effluent treatments.

Parameter	Municipal water	5% effluent	*p*	d.f.	*F*
n	26	29			
Wet weight (mg)	114 ± 4	112 ± 5	0.771	55	0.001
Fork length (cm)	2.1 ± 0.1	2.0 ± 0.1	0.011	55	4.718
Condition factor[a]	1.18 ± 0.02	1.32 ± 0.03	0.001	54	11.640
Cumulative mortality (%)	71.8	73.3			

Data expressed as mean ± standard error of the mean. Data was considered significant if $p<0.05$ using *t*-test. *p*: *p*-value; d.f.: degrees of freedom; *F*: *F*-statistic.
[a]Condition factor = $[(100\times\text{weight})/(\text{length}^3)]$.

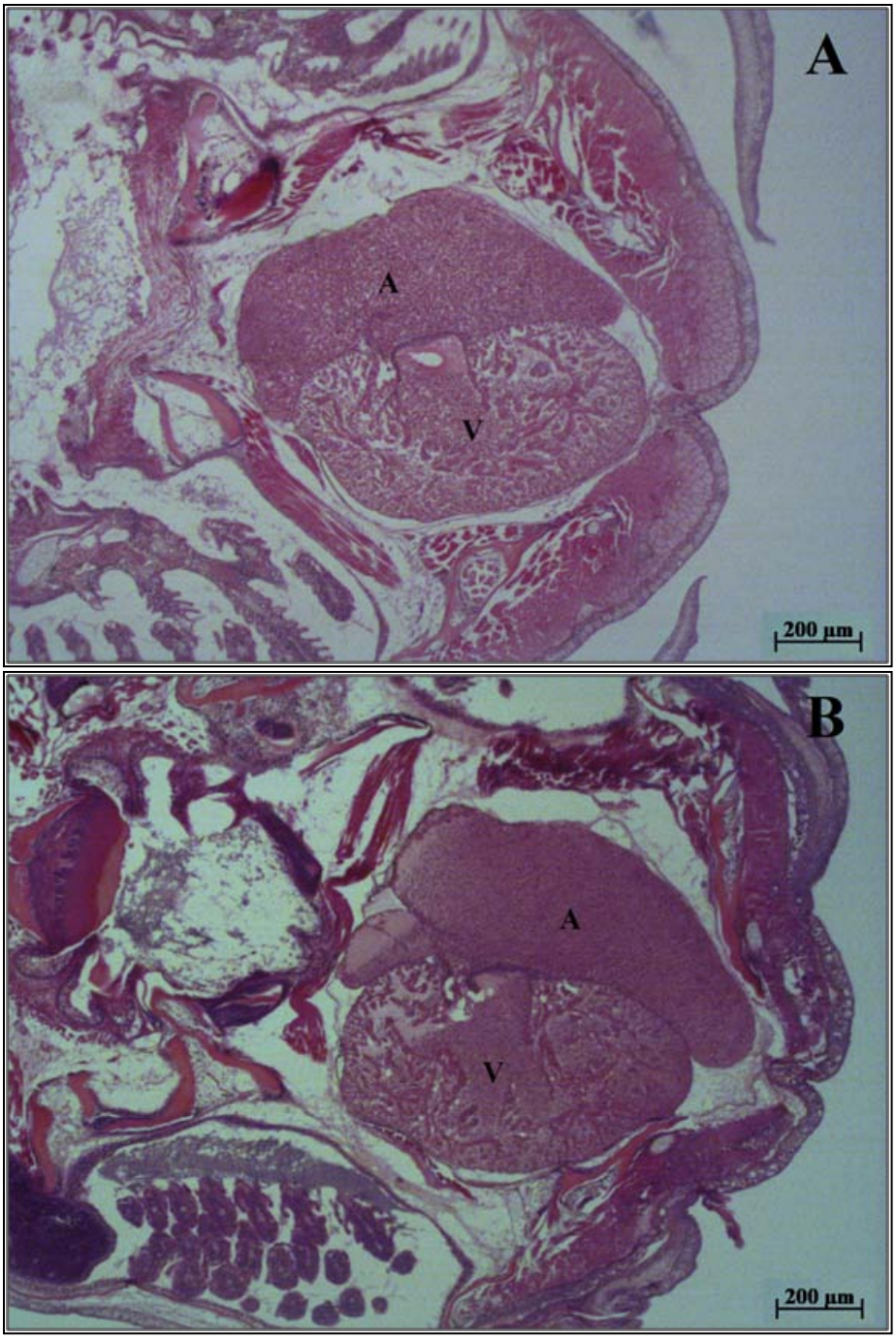

Figure 4.1 Representative images of hematoxylin/eosin stained sections of 60 day post hatch fathead minnow (*Pimephales promelas*) ventricle from reference (A; municipal water) and exposure (B; 5% effluent) treatments at Toxicology Centre, Saskatoon, Saskatchewan. A, atrium; V, ventricle. Images are shown at approximately 50× magnification.

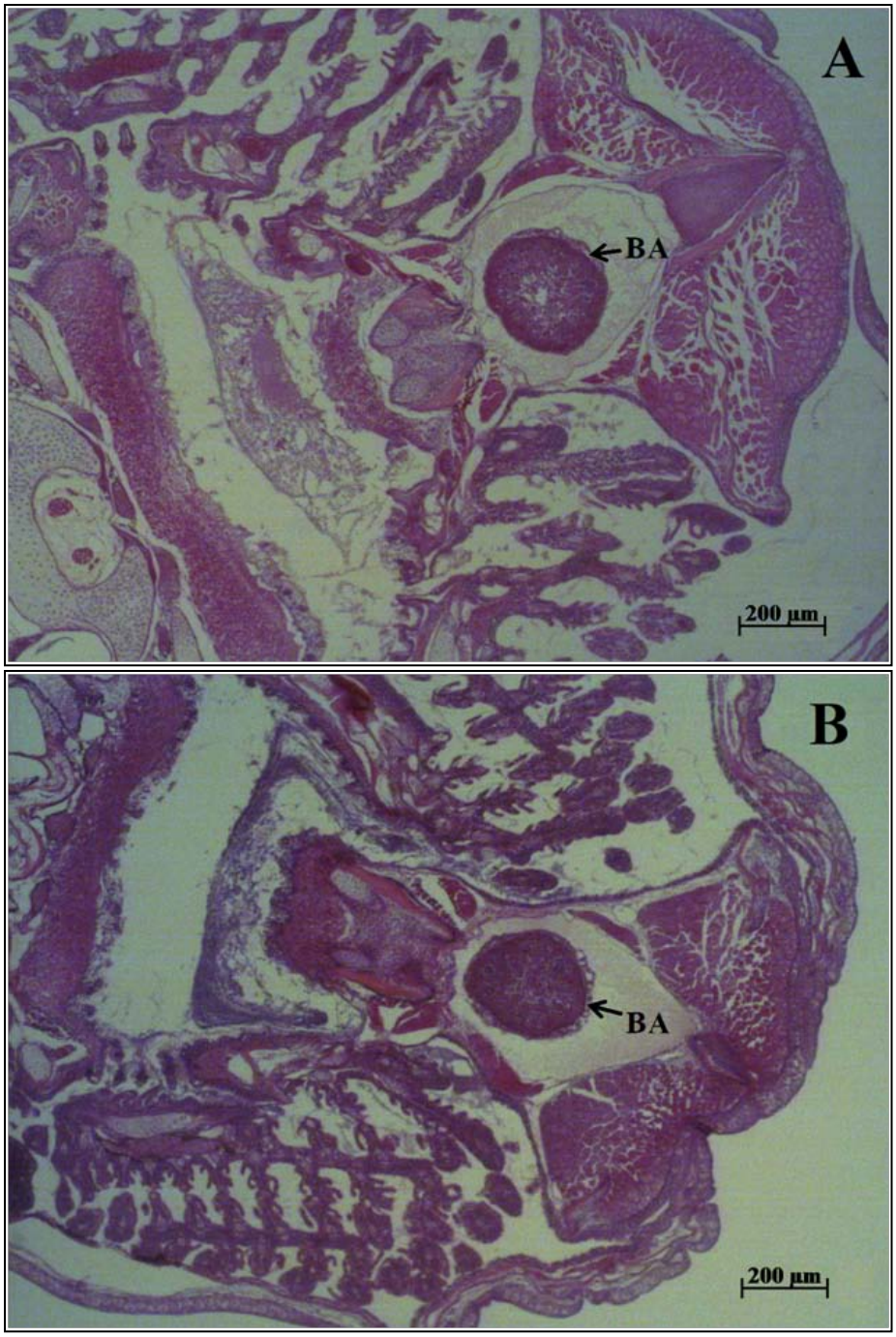

Figure 4.2 Representative images of hematoxylin/eosin stained sections of 60 day post hatch fathead minnow (*Pimephales promelas*) bulbus arteriosus from reference (A; municipal water) and exposure (B; 5% effluent) treatments at Toxicology Centre, Saskatoon, Saskatchewan. BA, bulbus arteriosus. Images are at approximately 50× magnification.

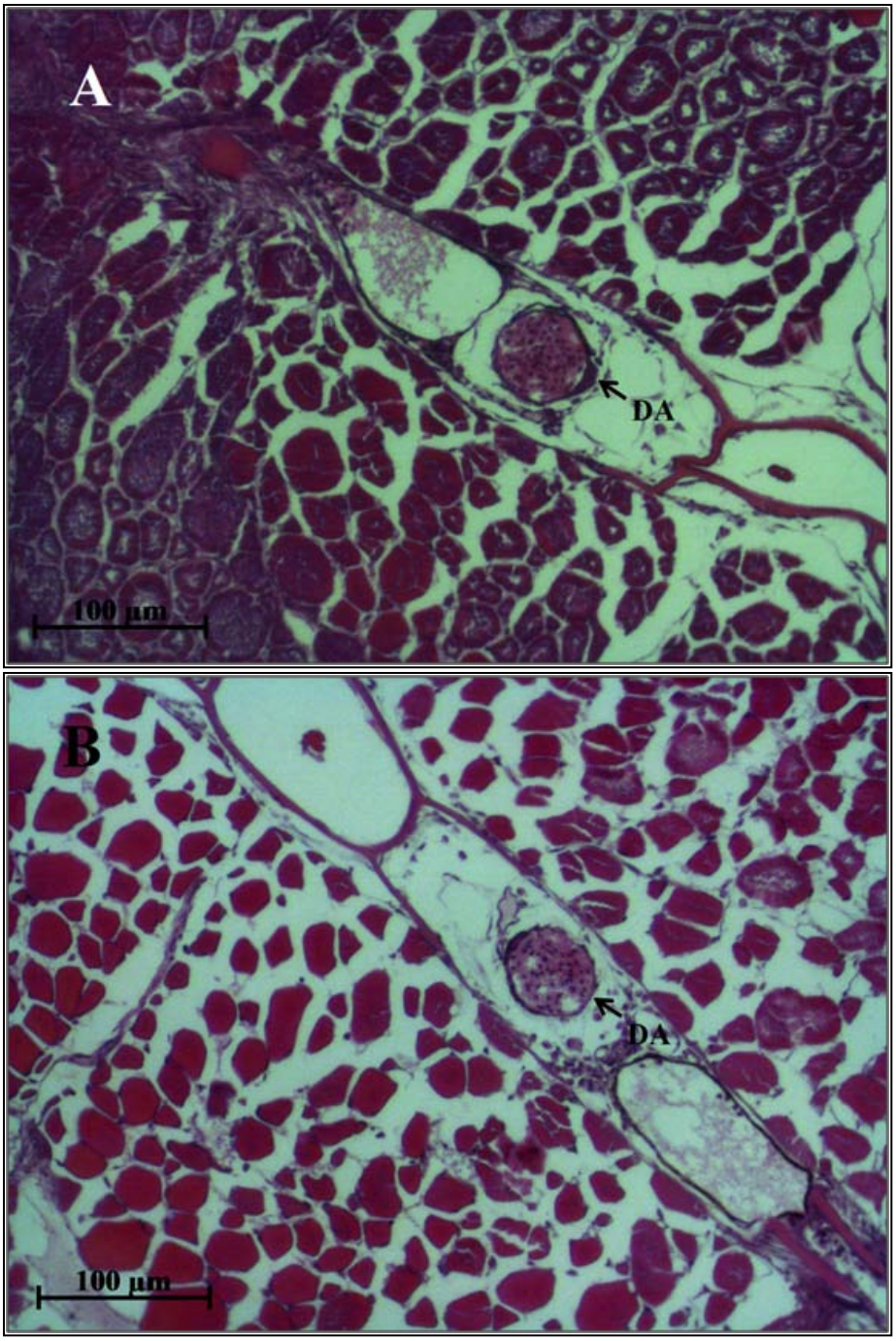

Figure 4.3 Representative images of hematoxylin/eosin stained sections of 60 day post hatch fathead minnow (*Pimephales promelas*) dorsal aorta from reference (A; municipal water) and exposure (B; 5% effluent) treatments at Toxicology Centre, Saskatoon, Saskatchewan. DA, dorsal aorta. Images are shown at approximately 200× magnification.

treatments (Table 4.4). Furthermore, no histological abnormalities were noted.

Critical swimming speed was significantly lower in fish from the exposure treatment ($p<0.001$) compared to control (Table 4.5). Similar results were obtained if critical swimming speed was analysed as absolute velocity (cm/s). There were no significant differences in tail beat frequency or tail beat amplitude between effluent exposed and control fish at 60 dph (Table 4.5).

Whole body glycogen was not affected by swimming ($p=0.121$ for swim factor in two-way ANOVA; 34 degrees of freedom; *F*-statistic 2.311; Figure 4.4A) or effluent exposure ($p=0.088$ for treatment factor; *F*-statistic 1.843). However, swimming had a significant effect on whole body triglycerides ($p=0.001$ for swim factor in two-way ANOVA; 35 degrees of freedom; *F*-statistic 14.132; Figure 4.4B). Specifically, whole body triglycerides were significantly lower in fatigued effluent exposed ($p<0.05$) and fatigued control fish ($p<0.01$) compared to corresponding non-fatigued fish. Effluent exposure did not significantly affect triglycerides compared to control ($p=0.744$ for treatment factor; *F*-statistic 0.108). There was no difference in whole body protein concentration in 60 dph FHM based on treatment or swimming (data not shown).

There were no significant differences in whole body CS or HOAD enzyme activity between fatigued and non-fatigued FHM from the same treatment (data not shown). As a result, enzyme activity data for both fatigued and non-fatigued fish from the same treatment were combined. Whole body CS activity was significantly lower in fish from the effluent exposed group ($p<0.01$ in *t*-test) compared to control (Table 4.6). In contrast, whole body HOAD activity was not significantly different between treatments.

Table 4.4 Ventricle diameter (*n*=8 fish per treatment) and bulbus arteriosus muscle diameter (*n*=8 fish per treatment; μm/g body mass), and dorsal aorta inner diameter (*n*=6-8 fish per treatment; μm/g body mass) of 60 day post hatch fathead minnow from municipal water and 5% Key Lake uranium mill (Saskatchewan, Canada) effluent treatments.

	Municipal water	5% effluent	*p*	d.f.	*F*
Ventricle	5272.7 ± 293.9	5651.1 ± 436.2	0.484	15	0.517
Bulbus arteriosus	1999.8 ± 131.2	2024.0 ± 174.1	0.913	15	0.012
Dorsal aorta	379.3 ± 32.7	383.2 ± 35.1	0.938	13	0.006

Data expressed as mean ± standard error of the mean. Data was considered significant if $p<0.05$ using *t*-test. *p*: *p*-value; d.f.: degrees of freedom; *F*: *F*-statistic

Table 4.5 Critical swimming speed (U_{crit}; *n*=19-21 fish per treatment), tail beat frequency (*n*=19-21 fish per treatment) and tail amplitude (*n*=19-21 fish per treatment) in 60 day post hatch fathead minnow from municipal water and 5% Key Lake uranium mill (Saskatchewan, Canada) effluent treatments.

Parameter		Municipal water	5% effluent	*p*	*F*
U_{crit} (BL/s)		6.18 ± 0.20	5.19 ± 0.13	<0.001	16.861
Tail beat frequency (Hz)		12.09 ± 0.19	12.00 ± 0.31	0.356	0.873
Tail amplitude (mm)	Left stroke	3.93 ± 0.21	4.07 ± 0.25	0.706	0.145
	Right stroke	3.95 ± 0.27	4.14 ± 0.25	0.660	0.197

Data expressed as mean ± standard error of the mean. 39 degrees of freedom for all parameters. Data was considered significant if $p<0.05$ using *t*-test. *p*: *p*-value; *F*: *F*-statistic

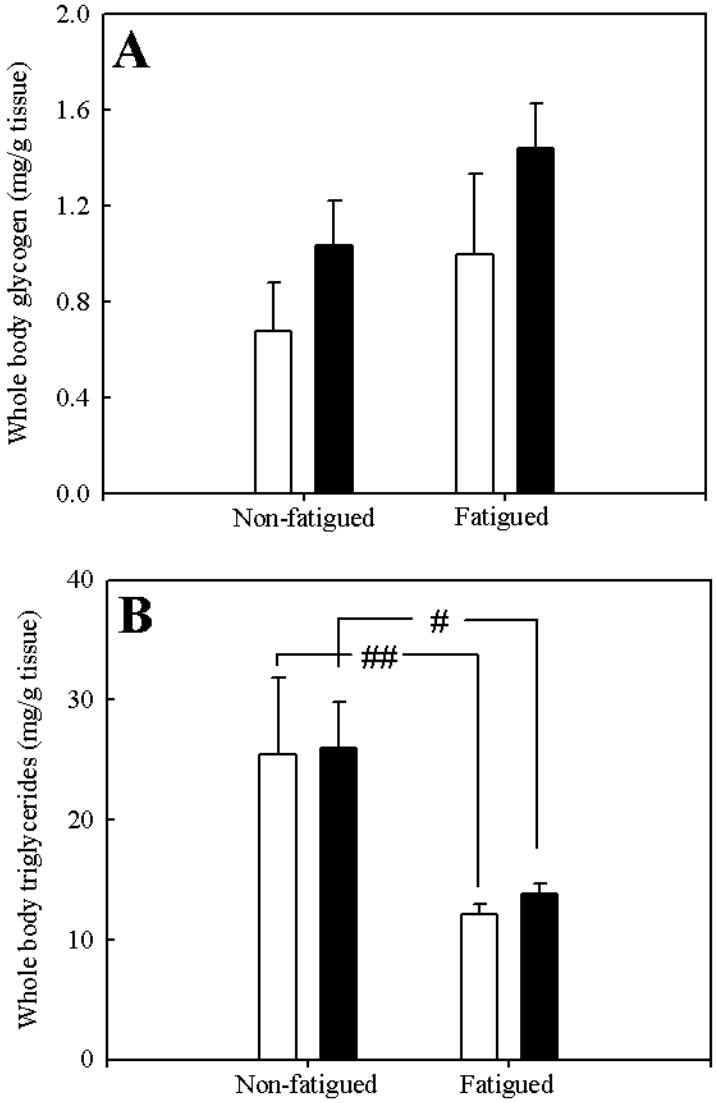

Figure 4.4 Whole body glycogen (A; *n*=8-10 fish per group) and triglycerides (B; *n*=8-10 fish per group) in 60 day post hatch fathead minnow (*Pimephales promelas*) from municipal water (control; open bars) and 5% effluent (exposure; solid bars) treatments. Fish in "Fatigued" groups were used in critical swimming speed tests, while fish in "Non-fatigued" groups were withheld from swim tests. For glycogen, p=0.139 for swim factor, p=0.184 for treatment factor in two-way ANOVA. For triglycerides, p=0.001 for swim factor, p=0.744 for treatment factor in two-way ANOVA. #p<0.05, ##p<0.01 compared to Non-fatigued fish from same treatment in Least Square Difference posteriori tests after two-way ANOVA. Data expressed as mean ± standard error of the mean.

Table 4.6 Whole body citrate synthase (CS; n=8 fish per treatment) and β–hydroxyacyl coenzyme A dehydrogenase (HOAD; n=7 fish per treatment) activities (International Units/g tissue) in mixed fatigued/non-fatigued 60 day post hatch fathead minnow (*Pimephales promelas*) from municipal water and 5% Key Lake uranium mill (Saskatchewan, Canada) effluent treatments.

	Municipal water	5% effluent	*p*	d.f.	*F*
CS	4.04 ± 0.31	2.64 ± 0.24	0.003	15	12.517
HOAD	0.49 ± 0.03	0.41 ± 0.03	0.083	13	3.585

Data expressed as mean ± standard error of the mean. Data was considered significant in $p<0.05$ using *t*-test. *p*: *p*-value; d.f.: degrees of freedom; *F*: *F*-statistic.

4.4 Discussion

The present study is the first to investigate swimming performance and energy homeostasis in laboratory raised fish exposed to uranium mill effluent. Overall, it was demonstrated that effluent exposure had significant effects on U_{crit} and aerobic capacity (CS activity) in juvenile FHM. Effluent exposed fish had significantly lower U_{crit} compared to control, which supports previous studies investigating metal and metalloid exposure and swimming performance (Wilson and Wood, 1992; Beaumont et al., 1995; Alsop et al., 1999; McGeer et al., 2000; Rajotte and Couture, 2002; Taylor et al., 2004). Effluent exposure did not appear to cause morphological effects that restricted tail beat frequency or tail beat amplitude, nor were cardiovascular changes evident. It is well established that reduced swimming ability correlates with lower maximum tail beat frequency (Bainbridge, 1958) and reduced cardiovascular ability (Farrell, 2007). Therefore, the results in the current study suggest that decreased U_{crit} may instead be attributed to some biochemical or metabolic effect of effluent exposure. However, ecological implications of altered swimming endurance in wild fish are not clear.

Ammonia negatively affects fish swimming performance at relatively low concentrations (Beaumont et al., 1995; Shingles et al., 2001; Wicks et al., 2002; Scott and Sloman, 2004; Tudorache et al., 2008a). Ammonia exposure probably reduces swimming performance by altering central nervous system and white muscle activity via alterations in ion and membrane charge balance (Randall and Tsui, 2002; McKenzie et al., 2003). As a result, ammonia has been shown to decrease maximum tail beat frequency in rainbow trout during U_{crit} tests (Shingles et al., 2001). Key Lake uranium mill effluent contains elevated ammonia, and concentrations in Delta Lake (downstream of the Key Lake uranium mill) average 0.6 mg/L (Golder Associates,

2008), which is similar to ammonia concentrations in the 5% effluent treatment in the present study. Therefore, it is plausible ammonia contributed to lower U_{crit} in effluent exposed fish, especially at higher swimming speeds for which white muscle is required. However, if this were the case, it is unclear why swim motion parameters were unaffected in the current study.

While some metals, such as aluminum (Wilson and Wood, 1992) or copper (Beaumont et al., 1995) impair swimming ability by directly affecting ionoregulatory or hematological mechanisms, exposure to certain trace elements or complex mixtures containing trace elements in wild fish can alter tissue metabolic ability (Levesque et al., 2002; Rajotte and Couture, 2002). In the present study whole body CS activity was significantly lower in effluent exposed FHM. In most fish species, swimming ability depends on overall aerobic metabolic scope, as activity up to 80% U_{crit} relies almost exclusively on oxidative metabolism (Webb, 1971a). Citrate synthase is a rate-limiting enzyme involved in the citric acid cycle, the impairment of which can significantly decrease aerobic adenosine triphosphate (ATP) production. Accordingly, lower oxidative phosphorylation levels would significantly reduce U_{crit} performance. Indeed, in one study environmental metal contamination lowered muscle CS activity in wild perch, which coincided with significantly lower U_{crit} (Rajotte and Couture, 2002). Alternatively, HOAD activity reflects tissue oxidative capacity to utilize lipids, the major muscle fuel source during aerobic activity (Magnoni and Weber, 2007). In the present study whole body HOAD activity was similar between treatment groups, implying that FHM from both treatments had comparable triglyceride catabolism during swimming. In support of this, whole body triglycerides were comparably lower in both treatment groups after swimming. These results highlight the importance of triglycerides as a metabolic fuel source during U_{crit} tests. Overall, the results presented here suggest 60 dph FHM exposed to 5% uranium mill effluent have the ability to swim comparably

to control fish until tissue ATP becomes limiting (possibly due to reduced CS activity), which leads to earlier fatigue. These data imply effluent exposure reduces U_{crit} (at least in part) by decreasing oxidative phosphorylation, but not lipid metabolism, during swimming.

Recent field studies at the Key Lake uranium mill have reported that a number of fish species exhibit altered energy stores compared to fish from reference lakes. Specifically, elevated liver triglycerides were reported in juvenile pike (*Esox lucius*), burbot (*Lota lota*), and in whole body spottail shiner (*Notropis hudsonius*) downstream of the Key Lake uranium mill (Bennett and Janz, 2007; Kelly and Janz, 2008), while juvenile pike exhibited elevated liver and muscle glycogen (Kelly and Janz, 2008). However, in the current study effluent exposure had no effect on juvenile FHM triglyceride or glycogen concentrations. It remains unclear why fish captured downstream of the Key Lake uranium mill have altered energy stores and that this was not observed in effluent exposed fish raised in the laboratory. However, species differences may have a role. Furthermore, in the present study food was equally available to juveniles in both treatments. Thus, any effluent effect on food quality or availability in the field was absent in this study. Nutrient enrichment in lakes downstream of the Key Lake uranium mill (*e.g.* increased nitrogen from ammonia) could increase food web productivity, thus contributing to elevated energy stores in fish. Conversely, Kelly and Janz (2008) demonstrated a lack of support for this hypothesis. Elevated energy stores in wild fish could also be related to water ionic strength. Most water bodies in the Key Lake region (including the reference sites) are characterised by very low ionic strength. However, addition of effluent may increase water ionic strength (as evidenced in the present study by elevated hardness and conductivity in the effluent treatment) enough to reduce osmoregulatory energetic requirements in wild fish, which could contribute to elevated energy stores (Shuter et al., 1989). Water conductivity differences between 5% effluent and

control treatments in the current study were far less than conductivity differences between lakes downstream of the Key Lake uranium mill and reference sites (Golder Associates, 2008). Thus, any metabolic advantage offered by higher ion content was minimized in the current study. Overall, water chemistry values for the 5% effluent treatment (except temperature that was modified for optimal FHM husbandry) were comparable with those reported in Delta Lake downstream of the Key Lake uranium operation (Bennett and Janz, 2007; Golder Associates, 2008; Kelly and Janz, 2008). In the control treatment however, water conductivity, hardness, and sulphate concentration were much higher than values observed in reference lakes near the Key Lake uranium mill. These differences may have also contributed to alterations in trace element body burden between treatments.

Earlier studies reported significantly elevated body burdens of a number of trace elements, most notably arsenic, molybdenum, selenium, and uranium in fish downstream of the Key Lake uranium operation (Pyle et al., 2001; Golder Associates, 2008). However, in the present study, FHM from the 5% effluent treatment had equal, or in many cases lower, trace element body burdens compared to fish from the control treatment. The discrepancy between laboratory and field results could be due to species differences, elevated water hardness in both treatments compared to field study reference sites (as ions related to water hardness can affect the bioavailability of some elements), as well as variation in effluent exposure route and duration. Wild fish downstream of the mill incur numerous routes of exposure (*e.g.* aqueous, dietary, sediment, maternal) to contaminants, over multiple generations, while in the present study effluent exposure only occurred over a short period of time. Furthermore, standard exposure routes for some elements were absent. Some elements, including arsenic, can be bioaccumulated directly from water, which may have contributed to the apparent elevated

concentrations in effluent exposed 60 dph FHM. Conversely, selenium accumulation is driven primarily by dietary exposure, which could explain similar selenium body burdens between treatments in the current experiment (Muscatello et al., 2008). Although brine shrimp fed to juvenile FHM were hatched in the same treatment, the brine shrimp themselves would also require dietary selenium exposure in order to transfer selenium to fish. Lastly, the present study is the first to expose FHM to diluted Key Lake uranium mill effluent in the laboratory and the degree of trace element accumulation through this exposure model is not yet established. Further research characterizing uranium mill effluent exposure in a laboratory setting is warranted.

Numerous studies report a toxicity-related decrease in condition factor in metal exposed fish (Lemly, 1993; Laflamme et al., 2000; Levesque et al., 2002; Muscatello et al., 2006). In contrast, in the present study condition factor was greater in effluent exposed FHM compared to control despite similar whole body energy stores between treatments. A similar study exposed larval FHM from 10 to 100 dph to diluted metal mine waste water (which contained similar trace elements as Key Lake uranium mill effluent) and also found effluent exposed fish had higher condition factor but similar whole body triglyceride levels (Driedger et al., 2010). Ammonia concentration in the exposure treatment was 0.069 mg/L, almost 10-fold greater than the reference treatment (Driedger et al., 2010). Indeed, FHM are known to tolerate a wide range of basic water quality characteristics and may respond differently to contaminant mixtures than more sensitive species, in this case with higher condition factor (Ankley and Villeneuve, 2006).

The toxic nature of selenium to freshwater oviparous organisms can lead to increased larval mortality (Holm et al., 2005; Muscatello et al., 2006). Extensive work has occurred at the Key Lake uranium mill documenting the bioaccumulative and teratogenic nature of the effluent discharged into the receiving environment (Muscatello et al., 2006; Muscatello et al., 2008;

Wiramanaden et al., 2010), but in the present study similar cumulative mortality rates occurred between treatments. However, overall survival rates were relatively low with greatest mortality occurring in all tank replicates within the second week after hatching, regardless of hatch date. Although many factors influence larval mortality in fish (which can occur at substantial rates in the wild), the high mortality during second week post hatch may be a normal consequence of fish development. In many species, mass embryo mortality occurs as larvae experience difficulties transitioning from yolk to free feeding stages (Sifa and Mathias, 1987). However, the switch from the eleutheroembryotic to larval stage in FHM occurs rapidly, approximately 24 hours after hatching (Belanger et al., 2010). Instead, larval mortality could be attributed to factors specific to food selection and larval morphology, rather than yolk absorption. For example, the cessation of mass larval mortality after 15 dph may have coincided with the ossification of important feeding structures, which permitted larger prey consumption and better nutrition (Anto and Turingan, 2010). Overall, relatively few larval studies report whole life cycle mortalities, and because of this the reasons for second week mass mortality are unclear, but do not seem to be related to exposure to the diluted effluent.

4.5 Conclusions

Effluent exposure negatively affected U_{crit} but not swim motion, cardiovascular morphology or triglyceride catabolism in laboratory raised juvenile FHM. While previous field studies show some fish species downstream of the Key Lake uranium mill have elevated energy stores, in the present study there was no evidence of elevated glycogen or triglycerides in whole body FHM between treatments. Reduced swim performance in fish from the 5% effluent treatment could be attributed to significantly lower CS activity in effluent exposed fish, or some other direct toxic effect of effluent exposure. The present study provides evidence that uranium

mill effluent exposure can alter metabolic status and impair swimming performance in laboratory raised fish. However, the biological significance of altered aerobic metabolism in these fish is unclear and further investigation on the effects of effluent exposure is warranted.

CHAPTER 5
5.0 GENERAL DISCUSSION

5.1 Overall conclusions

Previous studies report certain fish from metal contaminated environments have an altered metabolic status characterized by changes in tissue metabolic enzyme activity, stress response and swimming performance (Levesque et al., 2002; Rajotte and Couture, 2002; Audet and Couture, 2003; Taylor et al., 2004). Recent studies report altered bioenergetics in pike, burbot and spottail shiner downstream of the Key Lake uranium mill (Bennett and Janz, 2007; Kelly and Janz, 2008). Therefore, an investigation of the swimming performance and energy homeostatic effects in fish exposed to Key Lake uranium mill effluent was warranted. The purpose of the present thesis was to further characterize these effects of effluent exposure in fish collected downstream of the mill and in laboratory raised fish.

In summary, the first key observation of this study was that effluent exposure caused metabolic alterations in both captured wild and laboratory raised fish. However, effects on morphological, cardiovascular, and whole body trace element endpoints varied substantially between the field and laboratory experiments. The second key observation was that effluent exposure impaired U_{crit} in FHM in the laboratory experiment but not in wild shiner collected downstream of the Key Lake uranium mill, despite various metabolic and physiological alterations observed in effluent exposed shiner.

5.2 Energy homeostasis

5.2.1 Energy stores

Glycogen was elevated only in liver of both fatigued and non-fatigued shiner collected downstream of the uranuim mill at Key Lake (see Table 5.1 for summary of energy store

Table 5.1 Summary of the effects of effluent exposure (Treatment) and fatigue (Swimming) on muscle and liver energy stores in juvenile spottail shiner (*Notropis hudsonius*) collected mid June 2009 from reference (Yeoung Lake) and exposure (Delta Lake) sites at Key Lake uranium mill (Saskatchewan, Canada), and whole body energy stores in fathead minnow (*Pimephales promelas*) from municipal water and 5% Key Lake uranium mill effluent treatments. Treatment effects are shown under "Effluent exposed fish" columns and reflect differences in swimming metabolism between fatigued and non-fatigued fish exposed to effluent compared to reference/control water. Swimming effects are shown under "Fatigued fish" columns and reflect differences in swmming metabolism between effluent and control/reference water exposed fatigued fish compared to non-fatigued from the same exposure group. Results that differed significantly from the reference lake/control treatment (Treatment effects) or from non-fatigued fish (Swimming effects) are indicated by an arrow with the direction of change whereas those that did not differ are shown by a dash.

Energy store	Experiment	Tissue/Organ	Effluent exposed fish		Fatigued fish	
			Non-fatigued	Fatigued	Reference	Exposure
Triglycerides	Field	Shiner liver	–	↑	↓	–
	Field	Shiner muscle	–	–	–	–
	Laboratory	FHM whole body	–	–	↓	↓
Glycogen	Field	Shiner liver	↑	↑	–	–
	Field	Shiner muscle	–	–	–	–
	Laboratory	FHM whole body	–	–	–	–

results for all experiments in this thesis) compared to fish from the same swimming group from the reference lake. In contrast, no differences in energy stores were detected between control and effluent exposed FHM withheld from swimming tests in laboratory studies. It is interesting that in the field study both fatigued and non-fatigued shiner collected from downstream of the mill maintained elevated hepatic carbohydrate levels compared to fish from the reference sites, but the biological significance of this is unclear. Elevated glycogen stores may reflect different food web components or higher numbers of food items available in the exposure lake compared to reference lakes. This could explain why no difference in glycogen concentration was detected in effluent exposed FHM compared to control, given both treatments received essentially the same diet. Alternatively, mobilization of hepatic glycogen plays an important role in the acute stress response, and significantly elevated stores in shiner downstream of the mill could indicate some stress response alteration. Indeed, recent unpublished data showed shiner collected from the same exposure lake had impaired cortisol production upon acute stress stimuli (Hauck and Janz, unpublished). This data lends further support to previous studies reporting blunted stress responses in wild fish from metal contaminated environments (Brodeur et al., 1997; Laflamme et al., 2000; Levesque et al., 2002). Thus, fish downstream of the mill, as a result of impaired stress response, may fail to mobilize hepatic glycogen to the same degree as fish from reference lakes, which could result in the accumulation of hepatic glycogen. Alternatively, fish from the exposure site could have higher levels of glycogenesis compared to reference. Future studies could investigate these possibilities by comparing hepatic activities of enzymes involved in glycogen catabolism and anabolism in wild fish downstream of the Key Lake uranium mill and laboratory raised fish exposed to milling effluent.

It is interesting there were no differences in triglyceride stores in non-fatigued shiner or non-fatigued FHM in the laboratory experiment because elevated triglycerides are previously reported in certain fish downstream of the Key Lake uranium mill (Bennett and Janz, 2007; Kelly and Janz, 2008). Triglyceride stores are ecologically important for fish survival, especially for northern young-of-year fish (Pratt and Fox, 2002; Biro et al., 2004). The combination of reduced feeding and increased metabolic rate (due to detoxification processes) during winter is believed to significantly deplete energy stores required for fish survival (winter stress syndrome; Lemly, 1993). Indeed, some studies in fish demonstrate exposure to certain contaminants elevates metabolic rate (Farrell et al., 1998; McKenzie et al., 2007). The winter stress syndrome hypothesis was investigated in previous studies in fish species downstream of the of the Key Lake uranium mill, but results obtained demonstrated a lack of support for this hypothesis in the species studied from the Key Lake system (Bennett and Janz, 2007; Kelly and Janz, 2008). In this thesis effluent exposed fish from both the field and laboratory experiments did not have reduced energy stores, which would have been expected if extra energy had been diverted to detoxification processes. It is plausible that no difference was observed in the field study since shiner were sampled mid June, rather than early spring or late fall when seasonal effects could be most evident. This possibility could also apply to FHM in the laboratory study, which were reared at a constant, warm temperature with excess food supplied and thus were not subjected to seasonal changes.

5.2.2 Intermediary metabolism and changes in energy stores during activity

The results presented in this thesis lend further support to the importance of triglycerides during aerobic swimming. After swimming in the laboratory study, triglycerides were lower in whole body FHM from both treatments (Table 5.1). Similarly in the field study, swimming

lowered liver triglyceride levels in shiner from the reference lake, but not in shiner downstream of the Key Lake uranium mill (Table 5.1). Critical swimming speed is a measure of primarily aerobic swimming and the importance of triglycerides as a fuel source for this activity type, particularly in red muscle, has been investigated previously (Moyes and West, 1995; Magnoni and Weber, 2007). While swimming is primarily fuelled by intramuscular stores, adipose or hepatic stores could also be mobilized to fulfill active muscle fuel requirements, but the degree to which this occurs is unclear. Studies are needed investigating the kinetics of triglyceride and fatty acid mobilization from important tissue/organ (e.g. adipose, liver) stores to different muscle beds via the plasma in order to fully understand the differences between the whole body versus liver and muscle results in this thesis.

It is unclear why shiner downstream of the uranium mill in the field experiment had reduced ability to mobilize or use hepatic triglycerides during swimming (Table 5.1). If effluent exposure directly affected hepatic triglyceride mobilization or catabolism, it should have been evident in the laboratory raised FHM exposed to effluent. However, in the laboratory experiment whole body triglycerides decreased in both FHM from the 5% effluent and control treatments. It is plausible hepatic triglycerides were unchanged in effluent exposed FHM after swimming (similar to shiner in the field experiment collected downstream of the Key Lake uranium mill), but this was not detectable as whole body FHM concentrations were measured. Given previous reports of impaired cortisol production in wild fish exposed to metals from other contaminated systems (Brodeur et al., 1997; Laflamme et al., 2000; Levesque et al., 2002), stress response alterations could also be responsible for reduced hepatic triglyceride mobilization in shiner collected downstream of the mill in this thesis. Catecholamines, for example, are important regulators of lipolysis in fish, and are involved in regulating hepatic lipid mobilization during the

acute stress response (reviewed by Fabbri et al., 1998; van den Thillart et al., 2002). Fish exposed to Key Lake uranium mill effluent could have altered catecholamine production, akin to impaired cortisol production observed in previous studies examining mining operation contaminants (Brodeur et al., 1997; Laflamme et al., 2000; Levesque et al., 2002). Alternatively, effluent exposed fish could have altered adrenergic receptor expression in triglyceride-storing tissues. Overall, triglyceride metabolism in fish and the mechanisms by which it is regulated remain unclear. Further research investigating lipolysis in teleosts is warranted, which will help elucidate lipolytic alterations observed in liver from shiner downstream of the Key Lake uranium mill.

Given the aerobic nature of U_{crit} tests it is not surprising there were no statistically significant effects of swimming on shiner muscle, shiner liver, or FHM whole body glycogen stores (Table 5.1). Glycogen is primarily anaerobically metabolized in white muscle during burst activity or at swimming speeds greater than 80% U_{crit} (Webb, 1971a; Moyes and West, 1995). Therefore, ATP generated from anaerobic glycolysis likely accounted for relatively little of total ATP generated during U_{crit} tests. In one study eight minutes of forced exercise in Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), and rainbow trout resulted in a significant decrease in white muscle glycogen (McDonald et al., 1998). However, in the present study anaerobic activity in U_{crit} tests may have been insufficient to significantly decrease white muscle glycogen. While it is difficult to directly confirm anaerobic glycolysis occurred in fatigued fish, elevated plasma lactate in fatigued shiner from the reference lake suggests anaerobic glycolytic pathways were utilized. Lactate is primarily generated in white muscle and then mobilized to plasma during U_{crit} tests. Also, muscle constitutes the largest portion by weight of fish body mass. Therefore, it is plausible FHM whole body homogenate could be analysed in future studies

to determine lactate content. Greater whole body lactate in fatigued FHM would indirectly confirm that anaerobic glycolysis also contributed to anaerobic ATP production in FHM during U_{crit}.

Lastly, it was not surprising U_{crit} tests did not affect tissue or whole body protein content in any fish. Proteins play an important role in various metabolic processes (Jobling, 1994) and can serve as an important fuel source during swimming (Moyes and West, 1995). However, in most species it appears lipids are preferentially metabolised until stores are exhausted, *e.g.* in salmonids during extensive migratory swims and/or under starvation conditions (Moyes and West, 1995; Mommsen et al., 1999; Weber et al., 2003; Magnoni and Weber, 2007). Controlling for differences in feeding was not possible for shiner in the field experiment. In fact, it was initially suspected that variable nutritional status in wild shiner could overshadow effects of effluent contaminants on metabolic data, especially if feeding opportunities or nutritional status was significantly lower in fish from one lake versus the other. However, in both the field and laboratory studies there were no significant differences in tissue protein concentration between fatigued and non-fatigued fish. A previous study demonstrated muscle protein concentrations did not decrease in juvenile FHM deprived of food for 28 days, and even in juvenile rainbow trout (which were found to be more sensitive to food deprivation) muscle protein did not significantly decrease until 8 days of food deprivation (Weber et al., 2003). As a result, in this thesis it was highly unlikely that tissue protein concentrations would substantially change in either shiner in the field experiment or FHM in the laboratory experiment. Thus, lipid and carbohydrate stores, rather than tissue proteins, appear to have been the main energy sources utilized during swimming in both shiner and FHM.

This thesis is the first to investigate energy homeostasis combined with swimming performance in FHM and spottail shiner (both cyprinid species). These experiments demonstrated measurable changes in tissue energy stores were obtainable through U_{crit} tests in these species, and it is recommended future studies attempt to further characterize metabolic changes in small bodied fish to supplement currently salmonid-dominated data on exercise and intermediary metabolism. Testing red muscle energy homeostasis would be especially meaningful and provide important information on aerobic metabolic processes involved in swimming performance. However, limitations currently exist in small bodied fish, particularly those involving the extraction of sufficient tissue volumes for analysis and the limited resources available in field conditions.

5.2.3 Metabolic enzyme activity

There is little information on metabolic enzyme activities in cyprinids, and limited data specifically on CS or HOAD tissue activity in spottail shiner or FHM. For example, there is some information on CS activity in FHM brain tissue, but not liver or muscle (Georgiades and Holdway, 2007). Citrate synthase activity values reported in this thesis are comparable to activities reported in earlier studies in white muscle from different species of fish, but HOAD activities are generally slightly lower than previous reports (Leonard, 1999; Rajotte and Couture, 2002). Although muscle and whole body values for each enzyme are very similar between shiner and FHM, it is interesting that effluent exposure altered enzyme activity differently between the field and laboratory experiments (Table 5.2). The reason for the difference in observations is unclear but there are several possible explanations. First, white muscle accounts for the bulk of whole body mass. Thus, it seems likely that whole body homogenates should produce similar values to white muscle, making tissue differences an unlikely explanation. Secondly, enzyme

Table 5.2 Summary of results of citrate synthase (CS) and β–hydroxyacyl coenzyme A dehydrogenase (HOAD) muscle enzyme activities in juvenile spottail shiner (*Notropis hudsonius*) collected mid June 2009 from the exposure (Delta Lake) and reference (Yeoung Lake) sites at Key Lake uranium mill (Saskatchewan, Canada), and whole body activities in fathead minnow (*Pimephales promelas*) from municipal water and 5% Key Lake uranium mill effluent treatments. Results that differed significantly from the reference lake/control treatment are indicated by an arrow with the direction of change whereas those that did not differ are shown by a dash.

	Shiner muscle (Field study)	FHM whole body (Laboratory study)
CS	–	↓
HOAD	↑	–

activity variation between the two studies in this thesis could be attributed to species differences. Third, the difference in responses between the two experiments could be due to differences in the exposure regime/route and contaminants that fish accumulated in the field versus laboratory exposures.

While the effluent exposed fish showed enzyme activity differences in this thesis compared to reference/control fish, the degree to which effluent exposure was solely responsible for these changes is unclear. It has been demonstrated cold acclimation increases CS and HOAD activity in rainbow trout white muscle (Guderley and Gawlicka, 1992) and mitochondrial oxidation generally increases with cold acclimation (although the opposite has been observed in CS brain activity of certain fish; reviewed by Hochachka and Somero, 2002; Guderley, 2004; Georgiades and Holdway, 2007). In the field experiment the exposure lake from which shiner were collected is larger than the reference lake (269 ha versus 84.5 ha, respectively) and it is plausible these shiner habituated deeper, cooler spots within the exposure lake (Golder Associates, 2008). This difference could have partly contributed to elevated HOAD activity in the muscle of shiner downstream of the Key Lake uranium mill. A temperature- rather than effluent-related change is further supported by the lack of change in FHM HOAD activity since FHM in both treatment groups were kept in the same temperature-controlled room. However, if temperature was a key influence on enzyme activity, muscle CS activity should also be higher in shiner from the cooler exposure lake compared to the reference lake, which was not the case. Furthermore, a difference in CS activity was observed in FHM reared at the same temperature in the laboratory. Taken together, the results of this thesis do not support the hypothesis that temperature is the driving force changing enzyme activities. Instead, it appears alterations in

enzyme activities in both experiments are probably related to effluent exposure, but further studies are required to confirm these effects.

To further characterize biochemical-level effects of effluent exposure future studies could investigate other rate-limiting enzymes involved in intermediary metabolism. Specifically, muscle and liver cytochrome oxidase, lactate dehydrogenase, and phosphofructokinase activities would be of particular interest. Cytochrome oxidase is part of the mitochondrial electron transport chain for aerobic ATP production; lactate dehydrogenase mediates the interconversion of pyruvate and lactate in anaerobic glycolysis; and phosphofructokinase is also a rate-limiting enzyme involved in glycolysis that catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate (Hochachka and Somero, 2002). Some studies have shown metal contamination alters the activity of these enzymes in certain wild fish (Levesque et al., 2002; Rajotte and Couture, 2002; Audet and Couture, 2003; Lapointe and Couture, 2010). Other enzymes of interest that could also be investigated are pyruvate kinase (involved in the generation of pyruvate during glycolysis), creatine kinase (generates ATP from phosphocreatine), and carnitine palmitoyltransferase (mediates the transport of long-chain fatty acids into the mitochondria).

5.2.4 Intermediary metabolism and the acute stress response

Certain studies in wild fish exposed to environmental metals reported impaired cortisol production, which consequently prevented elevation of circulating glucose upon an acute stress stimulus (Laflamme et al., 2000; Levesque et al., 2002). In this thesis, similar impairment in the stress response could explain the failure of plasma lactate and hematocrit to change in fatigued shiner collected downstream of the Key Lake uranium mill. Unfortunately, it was not feasible to obtain FHM plasma samples due to their small size at 60 dph. As a result, direct comparisons

between the field and laboratory experiments were not possible. The results discussed here suggest future studies should investigate the effects of effluent exposure in primary and secondary acute stress response in wild and laboratory raised fish. Furthermore, it would be interesting to investigate intermediary metabolic endpoints in effluent exposed adult FHM from which liver, muscle and plasma endpoints could be analysed separately.

5.3 Swimming performance

5.3.1 Effect of effluent exposure on swimming performance

Critical swimming speed tests are shown to be sensitive indicators of contaminant exposure in a number of species and to various contaminants (Wilson and Wood, 1992; Beaumont et al., 1995; Alsop et al., 1999; McGeer et al., 2000; Rajotte and Couture, 2002; Hopkins et al., 2003; McKenzie et al., 2003; Taylor et al., 2004; Kennedy and Farrell, 2006; McKenzie et al., 2007). Critical swimming speed can serve as an ecologically relevant assessment of swimming compared to traditional endpoints, but its direct relation to fitness and survival in wild fish is not yet established (Plaut, 2001). For example, much like the present field study, some studies in wild fish from contaminated environments report no change in swimming performance despite evidence of metabolic alterations (Taylor et al., 2004; McKenzie et al., 2007). Furthermore, the majority of studies that document negative effects of contaminant exposure on U_{crit} were performed on laboratory raised and exposed fish (Plaut, 2001). In the current study, it is unclear if reduced U_{crit} in effluent exposed FHM, but not wild shiner, was due to differences in species sensitivity or exposure. Future experiments could investigate swimming performance and energy homeostasis in wild FHM from contaminated lakes or laboratory raised shiner exposed to effluent. However, neither are available (FHM are absent from aquatic systems in the Key Lake region, and shiner husbandry is difficult in the laboratory). Overall, this thesis

fails to support the use of U_{crit} tests alone as an indicator of contaminant exposure in wild fish, given the discrepancies between the laboratory and field experiment data. More research is required to elucidate sublethal effects of exposure on survivability and the ecological relevance of U_{crit} tests in contaminant exposed fish.

5.3.2 Utility and limitations of U_{crit}

Although U_{crit} is arguably a sensitive organism-level indicator of exposure in some fish in certain environments, there is disagreement in the literature regarding some elements of the U_{crit} test protocol. For example, many U_{crit} protocols employ +30 minute velocity increment steps (Brett, 1964; Wilson and Wood, 1992; Thorarensen et al., 1993; Kieffer et al., 1998; Shingles et al., 2001; McKenzie et al., 2007), which is not generally feasible for field studies. The limited growing season in northern Saskatchewan (which involves major biotic and abiotic changes over a short period of time) demands rapid metabolic and biosynthetic changes that could cause significant variability between fish tested earlier and later in the growing season. The present study demonstrated meaningful results are obtainable in comparative studies using only 5 and 3 minute velocity increment steps, given the intermediary metabolic endpoint changes observed between fatigued and non-fatigued fish. Furthermore, the U_{crit} values obtained for shiner and FHM (approximately 4 BL/s for shiner, 5-6 BL/s for FHM) are relatively consistent with salmonid U_{crit} values in tests that used velocity increments of longer durations (approximately 2-5 BL/s; Beamish, 1978; Hammer, 1995). By drawing heavily upon the works of Beamish (1978), Brett (1964) and Webb (1975), in his review on U_{crit} test protocols, Hammer (1995) concluded there is substantial evidence to suggest velocity increments should be at least 15-20 minutes in duration (and up to 60-75 minutes if oxygen consumption measurements are required). However, these studies primarily focus on salmonid swimming ability or evaluate non-salmonid species

that are generally much larger than the minnow species used in the present study. Indeed, many foundational swimming performance and U_{crit} studies primarily used larger salmonids as the test species (Bainbridge, 1958; Brett, 1964; Webb, 1971a, b). It is also important to note that many salmonids include long migratory phases in their life cycle, which could also affect U_{crit} performance and thus U_{crit} test protocol. Therefore, it is plausible shorter U_{crit} test velocity increments are suitable and possibly of greater ecological significance for non-salmonid minnow species (such as shiner or FHM). Indeed, one study tested the effect of velocity increment time duration in swimming silverside (*Menidia menidia*; approximately 6.6 cm body length) on U_{crit} using velocity increment intervals of 2, 5, 10, 20, 30, 45 or 60 minutes at a constant ~1 BL/s water velocity (Hartwell and Otto, 1991). Critical swimming speed values were similar for at velocity interval durations (range 9.7-9.2 BL/s), except for 30 and 45 minute intervals, which yielded slightly lower U_{crit} values (range 8.6-8.4 BL/s; Hartwell and Otto, 1991). Overall, this study demonstrated velocity interval duration during U_{crit} tests did not substantially affect final U_{crit}. As a result, standard U_{crit} protocols and guidelines that recommended +20 minute velocity intervals (which have for the most part been developed for research in salmonids) may not be suitable for smaller fish species and are difficult to perform in field studies.

Many swimming performance protocols include an 8-24 hr acclimation period in the swim tunnel prior to U_{crit} tests to allow fish to recover from handling and confinement stress (van den Thillart, 1986; West et al., 1993; Plaut, 2001; Tudorache et al., 2008b). This recovery period allows metabolic endpoints (such as circulating cortisol or glucose that would have increased during handling) to return to baseline levels. The concern is that shorter recovery periods may leave insufficient time for stress hormones and metabolites to return to baseline levels, which could then alter metabolic status before and during U_{crit} tests, as well as final U_{crit} values.

However, it has been demonstrated that U_{crit} values do not significantly vary in fish allowed to recover from handling stress for 0, 0.5, 1.0, 2.0, 4.0, 8.0, or 16.0 hours in swim chambers (Peake et al., 1997). Furthermore, similar to issues surrounding velocity increment duration in field-based swimming performance tests, time and resources in field studies are limited. It follows that in field studies an +8 hour acclimation period is impractical and for the most part unfeasible. Similarly, in the laboratory study in this thesis, multiple fish from the same hatch date required testing over one day using a single swim tunnel. As a result, it is important to note that the present study does not attempt to establish ecological baseline values, but rather is designed as a comparative study to characterize differences between fish from effluent exposed versus control or reference groups.

5.3.3 Alternative or supplementary tests to U_{crit}

A number of alternative swimming performance endpoints beyond U_{crit} have been developed that could also be considered for future studies. For example, there is growing evidence in support of the utility of back-to-back U_{crit} tests. In one study repeated U_{crit} tests 40 minutes apart in sockeye salmon (*Oncorhynchus nerka*) were a particularly sensitive means to detect environmental stressor effects, including to contaminants (Jain et al., 1998). Measuring repeated U_{crit} tests could provide a more ecologically relevant evaluation of both swimming and metabolism by providing supplementary information on the recovery metabolism of fish. Indeed, in back-to-back U_{crit} tests, wild chub (*Leuciscus cephalus*) from metal contaminated sites had lower U_{crit} and oxygen uptake in second U_{crit} tests compared to initial tests (McKenzie et al., 2007). Interestingly, there were no differences in U_{crit} and oxygen uptake between fish from clean and contaminated sites using only one U_{crit} test (McKenzie et al., 2007). Given the

metabolic effects observed in effluent exposed shiner and FHM in the present study, further research is warranted investigating swimming performance in repeated U_{crit} tests.

As discussed in Chapter 1 of this thesis, other swimming performance tests that could be considered include sustained or burst swimming tests. Burst tests, which measure primarily anaerobic capacity, would be particularly interesting in future studies, given that plasma lactate failed to increase after swimming while plasma glucose appeared to be elevated prior to swimming in shiner downstream of the Key Lake uranium mill. These tests could also be particularly ecologically relevant given that shiner and FHM tend to be schooling, non-migratory fish and their ecological role as key prey for various predators could demand greater levels of burst activity rather than sustained aerobic swimming.

Lastly, whole organism metabolic endpoints based on oxygen consumption (M_{O2}) could be used to supplement U_{crit} tests. These endpoints include resting (or routine) metabolic rate measured before swimming tests, or active metabolic rate, measured during swimming tests. There is evidence that contaminant exposure can increase metabolic rate (Farrell et al., 1998; McKenzie et al., 2007). Given the energy homeostatic effects observed in the present study in both shiner and FHM, investigating metabolic rate in effluent exposed fish in future studies is warranted.

5.3.4 Utility of tissue enzyme activity as an indicator of swimming performance

The utility of tissue enzyme activity as an indicator of swimming performance has been investigated, but results are, for the most part, inconclusive (Gibb and Dickson, 2002). The present study highlights the emerging utility of CS activity in comparative studies to predict impaired swimming ability in contaminant exposed fish. Activity was impaired in whole body FHM that was reflected as lower U_{crit}, while similar CS activity in shiner from both lakes was

reflected in similar shiner U_{crit}. As discussed earlier, muscle CS activity is known to positively correlate with tissue aerobic activity level (Dickson, 1995). Alternatively, this thesis supports the utility of HOAD activity as an indicator of triglyceride metabolism, but not U_{crit}. Fathead minnow had similar whole body HOAD activity between treatments, and whole body triglycerides correspondingly decreased with swimming in both treatments. However, muscle HOAD activity was elevated in shiner from the exposure lake compared to shiner from the reference lake, which may have related to altered hepatic triglyceride metabolism during swimming. Although triglycerides are a primary fuel for the aerobic portion of U_{crit} tests, this thesis does not support the use of muscle or whole body HOAD activity as a predictor of aerobic swimming ability.

5.4 Comparison of field and laboratory experiments

The majority of studies investigating contaminant effects on swimming performance are laboratory-based and characterize sublethal responses to single contaminants (Plaut, 2001). These studies commonly lack variables encountered in field studies, including predator-prey interactions, acquired contaminant tolerances, or variations in feeding and nutrient availability. As discussed earlier, other studies report no difference in swimming performance in wild fish exposed to contaminant mixtures (Taylor et al., 2004; McKenzie et al., 2007). This observation suggests U_{crit} tests alone may not be an ecologically relevant indicator of contaminant exposure in wild fish. It follows that individual and environmental variability encountered in field studies could reduce the sensitivity of swimming performance tests to contaminant exposure. As a result, until there is a better understanding of the ecological relevance of swimming performance tests, U_{crit} alone should not be used as an indicator of exposure, but rather supplementary to further tests.

There were considerable differences in trace element body burdens between the field and laboratory experiment, which may have affected swimming and metabolic endpoints (Table 5.3). Body burden differences could be related to a number of variables between experiments, including effluent exposure route, exposure duration, and species sensitivity. As discussed in Chapter 3, fish downstream of the Key Lake uranium mill incur exposure through various routes, as elevated trace elements are present in water, sediment, and dietary components (Golder Associates, 2008). As fish accumulate trace elements some metals or metalloids (such as selenium) are deposited in embryos, which can also serve as another exposure route to young fish. Conversely, in the laboratory experiment adult FHM breeding pairs were only exposed to effluent in water and diet over a limited exposure period (approximately one month), and subsequent juveniles were only exposed to effluent through water from hatch to swim tests (60 days). Differences in water hardness between experiments may have also contributed to discrepancies in trace element body burdens. Ions that contribute to water hardness have the ability to biologically interact with some available metals and metalloids and vice versa, which could alter whole body trace element accumulation in some fish. For example, dissolved strontium is known to interact with calcium uptake (Tabouret et al., 2010; Polak-Juszczak, 2011). It is plausible that in the field study shiner from the exposure lake had lower strontium concentrations due to elevated water hardness compared to the reference site. In the laboratory study, meanwhile, water hardness was elevated in both control and effluent treatments (compared to the reference lake in the field study), and thus strontium was comparable in FHM from both treatments. Furthermore, in the field experiment discharged effluent would be subjected to an array of environmental degradation and sequestration pathways that were otherwise absent in the laboratory study. Lastly, species differences could also have

Table 5.3 Summary of results of trace element body burdens of interest in whole body juvenile spottail shiner (*Notropis hudsonius*) collected mid June 2009 from the exposure (Delta Lake) and reference (Yeoung Lake) sites at Key Lake uranium mill (Saskatchewan, Canada), and fathead minnow (*Pimephales promelas*) from the 5% Key Lake uranium mill effluent treatments compared to the municipal water treament. Results that differed significantly from the reference lake/control treatment are indicated by an arrow whereas those that did not differ are shown by a dash.

	Shiner (Field study)	FHM (Laboratory study)
Arsenic	–	↑
Cadmium	–	–
Iron	–	↓
Manganese	↓	↓
Mercury	↓	–
Molybdenum	↑	↓
Nickel	–	↓
Selenium	↑	–
Thallium	↑	–
Uranium	–	↓

played a role in differences between experiments, altering trace element uptake, metabolism, and excretion between shiner and FHM.

Various studies have shown selenium causes morphological deformities in some oviparous organisms, including certain fish downstream of the Key Lake uranium mill (Lemly, 2002; Holm et al., 2005; Muscatello et al., 2006). It is therefore interesting that in the present study no deformities were observed in any shiner or FHM. This result could be due to a number of different factors. Species differences could have played a role and it is plausible the species used in the present study have lower selenium sensitivity compared to other species. For example, brook trout appear to be less sensitive to environmental selenium contamination than rainbow trout (Miller et al., 2009). Alternatively, it is plausible developmental deformities occurred in selenium exposed larvae in lakes downstream of the uranium mill. Deformities could have increased susceptibility to predation in wild larvae and juveniles, such that affected fish were removed from the population prior to the field experiment fish collection. Craniofacial or cardiovascular deformities could also have impaired feeding ability or reduced access to sufficient food sources in both the field and laboratory experiments. If this was the case in the laboratory experiment in this thesis cumulative mortality should have been greater in the 5% effluent treatment, but this was not observed. Alternatively, trace element analysis showed FHM from both treatments had comparable selenium concentrations, such that the lack of selenium-induced deformities may be the result of comparably low selenium accumulation with both treatments in this thesis. Overall, the present study demonstrates gross developmental terata characteristic of selenium exposure were, for the most part, absent in Key Lake milling effluent exposed shiner and FHM.

It is unclear if the enlarged ventricles in shiner downstream of the Key Lake uranium mill were due to developmental or post-developmental events. In humans ventricular hypertrophy can occur as a result of physiological stimuli, such as exercise training, or pathological stimuli, such as hypertension or myocardial injury (Hill and Olson, 2008). Similar (although relatively minor) effects occur in some fish with exercise training (Gamperl and Farrell, 2004). Enlarged shiner ventricles could therefore be the result of physiological growth (perhaps due to site differences in predation or food that demand greater cardiac capacity), pathological hypertrophy (as a result of some unidentified cardiovascular pathology) or some other developmental effect. In any of these scenarios one would expect to observe some effect on swimming performance given the importance of cardiorespiratory function during activity (Claireaux et al., 2005; Farrell, 2007). Environmental selenium exposure is known to cause cardiovascular effects in wild fish (Lemly, 2002). Indeed, in the present study enlarged ventricle size was only observed in shiner from the exposure lake, and fish from this lake had elevated selenium body burden compared to reference. Effluent exposed FHM had selenium body burdens similar to fish from the control treatment and no change in ventricle size was observed. Overall the significance of enlarged ventricle in shiner downstream of the Key Lake uranium mill is unclear but it did not appear to affect swimming performance.

5.5 Conclusions

The present thesis investigated effects of exposure to Key Lake uranium mill effluent on swimming performance and energy homeostasis in two small bodied fish species. Overall, swimming performance was negatively affected in 5% effluent exposed laboratory raised FHM, but not in wild shiner captured downstream of the Key Lake uranium mill. Energy stores were elevated in shiner collected from the exposure lake, supporting previous studies in wild fish from

the same lake, but this was not observed in the laboratory experiment. Effluent exposure caused variable effects on intermediary metabolism and metabolic enzyme activity between the field and laboratory studies. These effects probably played some role in reducing U_{crit} in effluent exposed FHM. Discrepancies in results between experiments are probably due, in part, to different routes and durations of effluent exposure between wild spottail shiner and laboratory raised FHM. Overall, Key Lake uranium mill effluent causes metabolic effects in both wild fish downstream of the mill and laboratory fish aqueously exposed to effluent, but the overall effects on wild fish survivability remain unclear.

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